HUMAN T-LYMPHOCYTE COLONY FORMATION IN VITRO

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Submitted for the degree of Doctor of Philosophy

University of Edinburgh

1985

DECLARATION

This thesis has been composed entirely by myself. The work described is either my own or the result of experiments carried out under my direct supervision.

Edinburgh, December 1984

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr Andrew Wyllie, for his enthusiastic support throughout this work. I am indebted to the members of staff of the immunopathology laboratory, especially Dr Edna Dewar and Miss Elizabeth Ramage, who provided invaluable advice and technical expertise. I acknowledge technical assistance from Mrs Fiona Evans, and Mr Roderick Murray who assisted in the work on Hodgkin's disease and T-cell leukaemia. I thank Dr Guy Besley for performing gel-filtration procedures on conditioned media from leukaemia patients. I also wish to thank Mr Peter Seidelin and Mr Christopher Rigg who carried out some of the experiments reported in this thesis, while working as B.Sc.hons students under my supervision.

Parts of this work were supported by grants from the Scottish Hospital Endowment Research Trust and the Melville Trust. During the first two years of this thesis I was supported by a Research Training Fellowship from the Medical Research Council.

Finally I wish to thank Professor Sir Alastair Currie for his continuous support and encouragement over many years.

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ABSTRACT OF THESIS (Regulation 6.9)

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Degree Ph.D. *Date* 20th December, 1984

Title of Thesis HUMAN T-LYMPHOCYTE COLONY FORMATION IN VITRO

Human blood mononuclear cells (MC) stimulated with phytohaemagglutinin (PHA) were shown to form lymphocyte colonies in agar. Colony cells were shown to be mature T lymphocytes by immunological typing.

Colony formation by blood MC and purified T cells in primary culture was enhanced by monocyte (MoCM) and lymphocyte conditioned media (LyCM). Proliferation of colony cells in secondary culture was wholly dependent on a factor present in LyCM (probably interleukin 2).

Using a mathematical approach (Kinetic analysis) to analyse cell interactions, it was shown that at least three interacting cells are involved in PHA induced, primary T-cell colony formation. Further analysis showed that the requirement for cell interactions could be substituted for by the inclusion of MoCM and LyCM into cultures. These findings are discussed in relationship to current concepts of interleukin mediated regulation of T lymphocyte proliferation.

Studies of leukaemic T-cells, from patients with T-cell chronic lymphocytic leukaemia (T-CLL) showed that colony formation by T-CLL cells was dependent on growth factors in a similar manner to colony formation by normal T-lymphocytes. It was also shown that T-CLL cells could not only respond to growth factors but were also able to produce normal or increased amounts of interleukin 2.

Colony formation by blood MC from patients with Hodgkin's disease was shown to be subnormal. Interleukin 2 production was also shown to be decreased. The reduction in colony formation was shown to correlate with alterations in blood T-cell subsets and in some cases to be secondary to impaired interleukin 2 production.

Finally, in a study of glucocorticoid-induced inhibition of T cell proliferation, it was demonstrated that inhibition of colony formation by methylprednisolone was the result of inhibition of growth factor production by accessory cells.

Use this side only

GENERAL INTRODUCTION

The normal function of the immune system depends upon controlled lymphocyte proliferation. Conventionally proliferation has been investigated using simple, short term, cultures of lymphoid cell suspensions stimulated with antigen or mitogens (Nowell 1976). The use of such cultures to study cell proliferation, however, has been recognised to have several disadvantages (Metcalf 1977). A major problem results from the heterogeneity of freshly isolated blood or tissue cell suspensions which contain a mixture of T and B cell subsets and non lymphoid cells such as monocytes. This makes precise monitoring and identification of the proliferating cell(s) and analysis of cellular interactions difficult (Metcalf 1977). This study of human T cell colony formation was initiated in order to develop a more satisfactory method for the analysis of lymphocyte proliferation that would overcome these limitations of suspension culture.

The advantages of colony forming assays over suspension cultures are well recognised (Metcalf 1977): They allow relatively easy characterisation of proliferating cells and allow more accurate measurement of cell proliferation than suspension cultures (Roper and Drewinko 1977, Maurer 1981). Colony formation has also been shown to be a powerful in vitro technique for the identification of regulatory factors controlling growth and differentiation of haemopoietic and

lymphoid cells (Metcalf 1977, Rosenszajn et al 1978, 1981). A further advantage of colony forming assays is that they appear to be more sensitive in detecting perturbations of lymphocyte proliferation resulting from disease (Wilson and Dalton 1976) or use of pharmacological agents (Roper and Drewinko 1977).

The aim of the present study was therefore to develop a colony forming assay for human T lymphocytes that would allow analysis of cell interactions leading to T cell proliferation by normal T lymphocytes and which could be utilised subsequently to investigate T lymphocyte proliferation in patients with abnormalities of T cell function.

The results presented in the first chapter of this thesis deal with the establishment of the colony forming assay and the characterisation of the colony forming cell. In the second chapter the cell interactions and growth factor requirements for colony formation are analysed. In chapter 3 the information gained from studies of normal T cell colony formation is applied to the investigation of abnormalities in T lymphocyte function in patients with Hodgkin´s disease and T cell chronic lymphocytic leukaemia. In chapter 4 analyses of the mechanism of glucocorticoid inhibition of lymphocyte proliferation are described.

CHAPTER 1

CULTURE REQUIREMENTS AND PHENOTYPIC CHARACTERISTICS OF HUMAN T-CELL COLONIES IN VITRO.

SUMMARY

In this chapter preliminary investigations of PHA induced T cell colony formation are described. Two methods allowing colony formation were studied; a single step procedure in which freshly isolated blood mononuclear cells were plated directly into agar and a two step procedure in which mononuclear cells were preincubated in suspension with PHA before plating into agar. Comparison of these two methods showed that single step cultures offered advantages over the two step cultures including a higher plating efficiency and ease of harvesting of colony cells for further studies. Colony cells from these cultures were shown to be mature T-lymphocytes by immunological typing.

INTRODUCTION

The first description of human T-cell colony formation was provided by Rosenszajn et al in 1975. This group described a two step culture method which required preincubation of blood mononuclear cells (MC) with PHA in suspension for 12-24 hours, followed by plating of cells in a soft agar overlayer

onto an agar base in which PHA was present. Similar two step cultures were subsequently succesfully used to demonstrate colony formation by other groups (Fibach et al 1976, Wilson and Dalton 1976, Dao et al 1978). Initial attempts to grow T-cell colonies by directly plating MC into agar were unsuccessful (Rosenszajn et al 1975, Gerassi and Sachs 1976). However modifications of the culture methods were later described that allowed T-cell colony formation to occur. To obtain colony formation in single step cultures it was found to be necessary either to plate cells at a relatively high cell concentration (Riou et al 1976, Goube deLaforest et al 1979a), or to include autologous or sheep erythrocytes in the cultures (Gerassi and Sachs 1978, Claesson et al 1977a). More recently leucocyte feeder layers or conditioned media have been used to support single step colony formation (Foa and Catovsky 1979, Gelfand et al 1981). (TABLE 1A).

Colony cells harvested from PHA induced colonies have been shown to posess morphological features typical of lymphocytes undergoing blastic transformation and to form rosettes with sheep erythrocytes (E- rosettes). They do not express surface Ig or exhibit cytochemical staining characteristics of myelo-monocytic cells i.e. phenotypically they are T-cells (Rosenszajn et al 1975, Riou et al 1976, Claesson et al 1977a). Gelfand et al (1981) have shown that colony cells express the T cell antigen OKT3 and also expresS HLA-DR antigens characteristic of activated T-cells.

TABLE 1.A TECHNIQUES USED IN T-CELL COLONY FORMATION

Culture technique	Plating concentration	Plating efficiency	Reference
Two step cultures	6*10^5/ml	0.05%	Rosenszajn et al 1975
Two step cultures with autologous erythrocytes	6*10^5/ml	1.0-2.0%	Gerassi and Sachs 1976, 1978
One step cultures with thin agar overlayer	10*10^5/ml	0.4%	Riou et al 1976
Two step cultures and one step cultures with sheep erythrocytes	1*10^4/ml	5.0% 1.0%	Claesson et al 1977a,b
One step cultures with leucocyte feeder layer	1*10^5/ml	0.2%	Foa and Catovsky 1979
One step cultures in methylcellulose with conditioned media feeder layers	5*10^5/ml	0.8%	Gelfand et al 1981

The above data were abstracted from published work cited in the references column

Early studies of T-cell colony formation demonstrated that the colony forming cell precursor is an E-rosetting small lymphocyte, responsive to PHA in both liquid and agar culture (Claesson et al 1977a, Swart and Lowenberg 1980). Recently studies of colony formation have shown that T colony forming cell precursors may include not only an E- rosette positive, OKT3 positive T cell but also an E-rosette negative, OKT3 negative T cell precursor (Spitzer et al 1982).

The proportion of blood mononuclear cells that give rise to colonies has been variably reported as between 0.05% (Rosenszajn et al 1975) and 5.0% (Claesson et al 1977a) with other groups reporting intermediate values. These differences in plating efficiency are probably attributable to differences in the culture methods (summarised in table 1.A). Factors affecting plating efficiency have been found to include:

1) The number of cells plated and the cell concentration (Gerassi and Sachs 1976, Claesson et al 1977a, Goube de Laforest et al 1979a, Gelfand et al 1981).

2) The inclusion of red blood cells in cultures (Claesson et al 1977a, Gerassi and Sachs 1978, Woods and Lowenthal 1981).

3) The inclusion of leucocyte feeder layers or conditioned media in cultures (Claesson et al 1977b, Zeevi et al 1977, Gerassi and Sachs 1978, Gelfand et al 1981).

4) Other modifications of the culture medium; e.g. source of serum, concentration of certain amino acids, addition of

defined serum proteins such as transferrin, nature of the mitogen and mitogen concentration (Sachs 1978, Tice and Davey 1983).

In view of these reported variations in culture conditions and plating efficiencies, it was therefore necessary to establish, in preliminary studies, suitable conditions for colony formation in our laboratory. The results in this chapter describe the relative plating efficiencies of one step and two step cultures, the effect of varying the cell concentration, the effect of red blood cells in cultures, the serum requirements and the optimal mitogen concentration. In addition evidence is presented which confirms that the colonies growing in agar were of T-lymphocyte derivation. The effects of leucocyte feeder layers and conditioned media are dealt with in chapter 2.

MATERIALS AND METHODS

BLOOD MONONUCLEAR CELLS

Blood was obtained by venepuncture and added to sterile plastic universal containers, containing either heparin (10units/ml final concentration) or E.D.T.A. (2% v/v final concentration). Blood mononuclear cells (MC) were isolated by centrifugation over Ficoll Hypaque (Habeshaw and Young 1975). After centrifugation the plasma was removed, and kept if required,and the MC removed from the plasma-Ficoll interface.The MC were then washed twice in RPMI-1640 and resuspended at the required cell concentration.

COLONY FORMATION

Colony formation by blood MC was assessed using both a single step culture method,in which MC were plated directly into agar after isolation , and a two step method, in which MC were preincubated in suspension culture before plating in agar.

Single step colony formation: This technique is a modified version of a method described initially by Riou et al(1976).

Standard culture method:

Blood MC were suspended at cell concentrations between

0.625- 10.0x10^6/ml in 0.3% agar (Bacto-agar, Difco laboratories) in RPMI 1640 containing 15% autologous plasma,unless otherwise stated.This cell suspension was pipetted onto an underlayer of 0.5% agar in RPMI 1640 containing 15% autologous plasma and PHA (phytohaemagglutinin M, Difco laboratories). Unless otherwise specified PHA was added to give a 1% v/v concentration in the agar underlayer (i.e 20 microlitres of PHA-M diluted 1/10 were added to 200 microlitres of agar underlayer). Cultures were normally carried in 17mm diameter wells in Linbro multiwell plates (24x17mm diameter, flat bottomed wells, Flow laboratories); in these experiments 40 microlitres of cells in 0.3% agar were added to 200 microlitres of 0.5% agar underlayer. In some experiments washed autologous red blood cells were added to the underlayers at concentrations between 0.5-2.0%. Cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO2 in air.

Mass cultures:

These "mass cultures" were used to obtain colony cells in large numbers for cell phenotyping . MC were cultured in 90mm diameter petri dishes :1.0ml of cell containing overlayer was added to 6.0ml of underlayer. After 5- 7 days incubation, colonies were harvested by gently pipetting 1.0ml of RPMI 1640 onto the cultures. This caused the colonies to detach from the agar, and colonies could then be harvested by aspirating the medium. Cytospin preparations were then made from colonies

without further treatment. In order to obtain single cell suspensions harvested colonies were in some cases washed twice in TC199 and disaggregated by refluxing through a fine pasteur pipette. Cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO2 in air.

Colonies, defined as aggregates of greater than 40 cells, were counted after 6-7 days incubation, using x40 magnification light microscopy. Three fields were examined in each 17mm well and the number of colonies present within a ruled eye-piece grid were counted. As each grid area was equal to 1/20th of the total well area the total number of colonies/well could be estimated :

Total colonies/well= colonies in 3 fields x 20÷3

Colony size (i.e. the number of cells per colony) was measured either by direct examination of cultures at x100 magnification or by examination of photomicrographs.

Two step colony formation:This technique is modified from a method described by Rosenszajn et al (1975).

Five million freshly isolated blood MC were first preincubated in 5.0ml RPMI 1640 containing 10% autologous plasma, and 1%v/v PHA, for 18hours. The cells were then harvested by centrifugation, washed three times in RPMI 1640 , disaggregated by repeated passage through a fine pasteur

pipette, and resuspended in 0.3% agar, containing 10% plasma in RPMI 1640, at the required cell concentration. One ml of this suspension was then pipetted onto an one ml of agar underlayer, 0.5% agar in RPMI 1640 with 10% plasma and 1%v/v PHA, in a 35mm diameter petri dish. Cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO2 in air.

Colonies were counted after 5 days incubation by counting all the colonies (aggregates of more than 40 cells) present in the petri dish.

CELL PHENOTYPING

MC and cells harvested from colonies were characterised using light and electron microscopy, enzyme cytochemistry, immunohistochemistry, immunofluorescence, immunoperoxidase and rosetting procedures.

Cytospin preparations were made from cell suspensions (MC and colony cells) using a Shandon Mark II cytocentrifuge and were stained with May-Grunwald-Giemsa for routine morphological examination. Cytospin preparations were also stained for acid phosphatase, non specific esterase (alpha naphthyl acetate esterase) and chloroacetate esterase (Yam et al 1971). For electron microscopy, cell suspensions (10 million cells) were pelleted in plastic conical centrifuge tubes, the supernatant drained off, and the cells then

resuspended in 5-10mls of 2.5% glutaraldehyde in cacodylate buffer. Cells were then processed routinely for electron microscopy.

Immunological studies

Cell suspensions and cytospin preparations were stained with a panel of anti T and B-cell and anti monocyte antibodies (OKT3, OKT4, OKT6, OKT8, OKT11, OKM1-Ortho diagnostics, MO2, B1-Coulter, anti HLA-DR/DA6.231 and anti IgM/DA6.127 donated by Dr K.Guy) to demonstrate cell surface and cytoplasmic immunoglobulin and other cell antigens.

Immunofluorescent staining of cell suspensions with monoclonal antibodies

Aliquots of $0.5\text{-}1.0\text{x}10^6$ cells were pelleted in 12x7mm glass tubes and the supernatants removed. Ten microlitres of antibody at the appropriate concentration (diluted in phosphate buffered saline, pH 7.2 containing 2% human serum albumin [PBS- 2%HSA]) was then added to the tube and the cells were resuspended, and incubated on ice for 30minutes. Cells were then washed twice in ice cold PBS and the supernatants drained off. Twenty microlitres of fluorescein conjugated goat anti mouse immunoglobulin (FITC- GAMIg, Ortho diagnostics) at a 1/40 dilution in PBS-2%HSA was added and the cells resuspended and incubated on ice for a further 30 minutes. The cells were then washed twice in PBS and resuspended in 0.1ml of PBS.

A drop of this cell suspension was placed on a slide, covered by a cover slip and examined by immunofluorescence. The percentage of fluorescent cells was determined by counting at least 200 cells. Controls stained with only the second layer antibody (FITC-GAMIg) were also routinely examined. These controls usually showed less than 1% positive cells and never showed more than 5% positive (i.e. non specifically staining cells).

Demonstration of cell surface Immunoglobulin (SIg)

Cell suspensions were stained for SIg either by an indirect immunofluorescence procedure using rabbit anti human IgM as first layer antibody and FITC- conjugated goat anti rabbit antibody as the second layer antibody (Habeshaw and Young 1975) or by direct immunofluorescence using fluorescein (FITC) or rhodamine (TRITC) conjugated Fab2-sheep anti human kappa or lambda chain antibodies (Kallestad). In both direct and indirect procedures cells were first incubated at $1x10^6$/ml in TC199, at 37^oC, for 45 minutes, then washed once and resuspended at $10x10^6$/ml in PBS. Aliquots of $1x10^6$ cells were then incubated with antibody at the appropriate dilution (1/80 for rabbit anti human IgM and 1/40 for FITC and TRITC- conjugates), for 30 minutes at 4^oC and then washed twice in PBS. In the indirect procedure the second layer antibody (FITC-GAR) was then added at 1/40 dilution and incubated for 30 minutes at 4^oC, following which the cells were again washed twice in PBS. In order to determine the percentage of cells

with SIg at least 200 cells were counted using the fluorescent microscope.

Cytoplasmic Immunoglobulin (CytIg)

Cytospin preparations were fixed in 90% ethanol for 90 seconds. CytIg was detected using the same antibodies, at the same concentrations, as were used for SIg. The cytospins were treated with antibody for 30 minutes , at room temperature, in a humidified box. They were then washed twice in PBS. For indirect immunofluorescence, cytospins were then stained with second layer antibody (GAR-FITC) for a further 20 minutes, and then washed twice in PBS. Cytospins were mounted in glycerol saline and examined by immunofluorescence.

Immunoperoxidase staining of cytospin preparations with monoclonal antibodies.

The following procedure was used to stain cytospin preparations of intact colonies by an indirect immunoperoxidase staining technique.

1.Cytospins fixed in acetone for 30 seconds.

2.Washed in PBS(pH 7.4) for 5 minutes.

3.Treated with normal rabbit serum (NRS), 20%NRS in PBS, for 10 minutes, and drained.

4.Treated with monoclonal antibody at the appropriate dilution for 30 minutes. All antibodies, except DA6.231 and anti kappa and lambda, were used at 1/20 dilution. DA6.231 was used at 1/80, and anti kappa and anti lambda were used at 1/2000. Antibodies were all diluted in PBS- 20%NRS.

5.Washed in PBS for 5 minutes, twice .

6.Washed in PBS- 20%NRS for 10 minutes, once.

7.Treated with peroxidase conjugated rabbit anti mouse immunoglobulin (Dakopatts),(Peroxidase- RAMIg), at a 1/20 dilution for 30 minutes.

8.Washed in PBS for 5 minutes, twice.

9.Treated with Graham and Karnovsky's standard DAB solution, containing .01M Imidazole. Staining was controlled microscopically; with anti kappa and lambda staining times were about 5 seconds; with other antibodies staining times were about 5 minutes.

10.Washed in tap water.

11.Counterstained with a 1% aqueous solution of toluidine blue.

12.Dehydrated through alcohol, cleared in xylene, and mounted using a synthetic resin.

Rosetting

Rosetting was used to demonstrate T- cells with sheep red blood cell receptors (E-rosettes), B-cells with mouse red blood cell receptor (MRBC- rosettes), and cells with FcIgG, FcIgM, and Complement receptors by rosetting with IgG, IgM, and Complement coated Ox red blood cells (OxEAG, OxEAM, OxEAC). The methods used have been fully described (Habeshaw and Young 1975, Stockdill et al 1983).

RESULTS

Culture requirements and morphological observations of T-cell colony formation by blood MC.

Freshly isolated blood MC formed colonies when plated directly into agar, in single step cultures, only if PHA was present in the culture underlayers. Colony formation was maximal at a 1%v/v concentration of PHA (FIG 1.1). In two step cultures MC formed colonies only if PHA was present both in the liquid preincubation phase and subsequently in the agar underlayer.

In one step cultures freshly plated blood MC were dispersed as single cells in culture overlayers. Sequential observations of cultures showed that small clusters of 4-8 cells were visible on the surface of the agar within two days of the initiation of cultures; these progressively increased in size until day 5 when colonies (greater than 40 cells) were clearly visible on the agar surface. At day five, in addition to colonies, smaller cell clusters (4- 40 cells) and enlarged single cells could also be seen both on the agar surface and occasionally deeper in the agar.(PLATES 1.1, 1.2). The appearance of colonies , after day 5, usually remained static for the next two to three days following which colonies began to degenerate. Two step cultures plated after preincubation with PHA consisted of both single cells and small clumps of cells. In these cultures colonies developed within the agar as irregular aggregates with only a small number of well defined rounded superficial colonies that predominated in the single step cultures

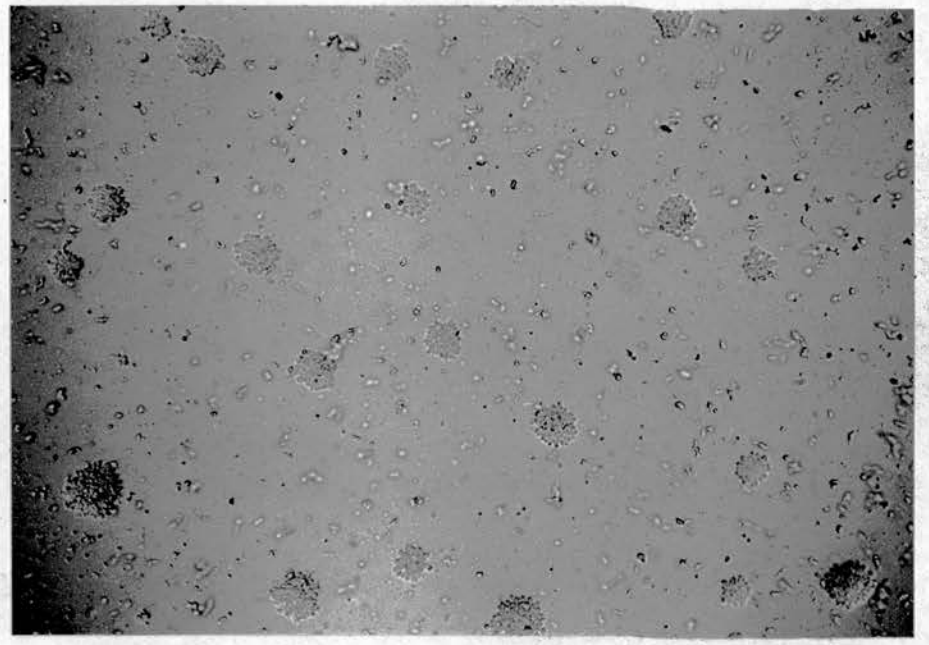

PLATE 1.1 T-LYMPHOCYTE COLONIES
Light microscopy. (original magnification x 40)

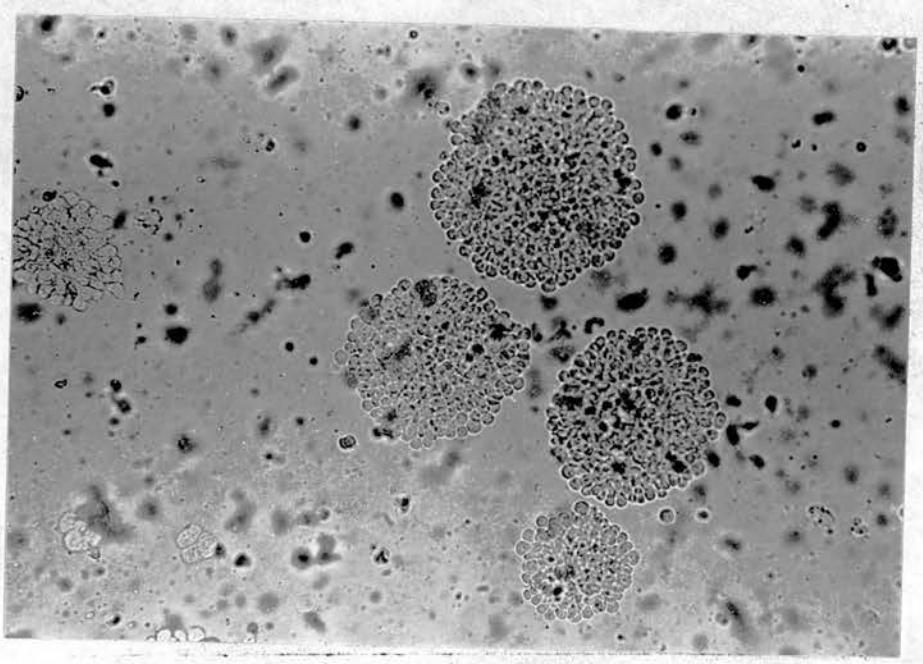

PLATE 1.2 T-LYMPHOCYTE COLONIES
Light microscopy. (original magnification x 100)

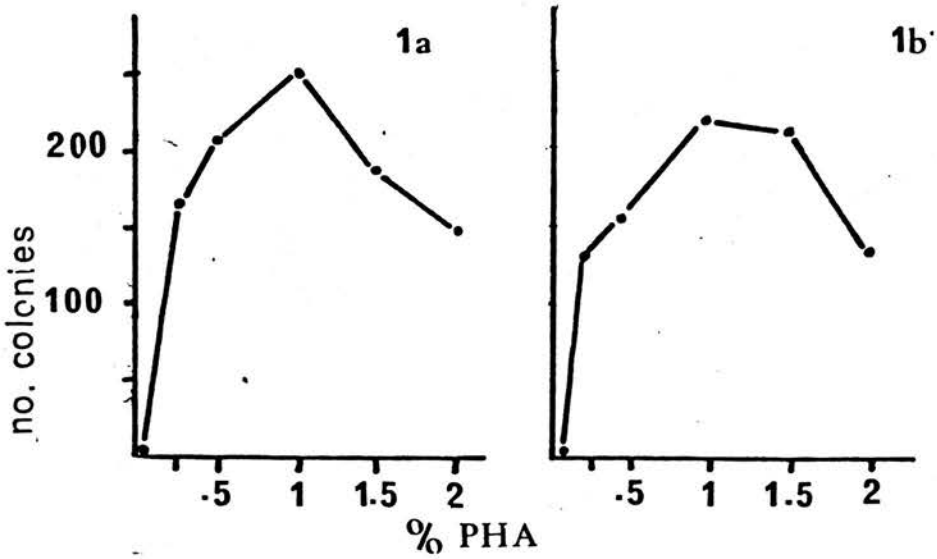

Figure 1.1: The effect of PHA concentration on colony formation in single step cultures.

1a: standard culture method

1b: culture with 1% RBC in underlayer

Points are the mean values of quadruplicate cultures of blood mononuclear cells, (2×10^5 cells/well) from a single donor. Similar results were obtained using mononuclear cells from other donors.

In both one step and two step cultures colony formation was greater when autologous plasma or human serum (pooled heat inactivated normal human serum) were used rather than foetal calf serum. No colonies grew in the absence of serum (TABLE 1.1).

TABLE 1.1

THE EFFECT OF DIFFERING SERUM SOURCES ON COLONY FORMATION

	Number of colonies		
	One step cultures		Two step
	Standard underlayer	RBC underlayer	culture
15% Autologous Plasma	92±5	112±12	199±17
15% Pooled normal heat inactivated human serum	99±11	101±13	169±16
15% Foetal calf serum	10±8	50±8	24±9

Figures are mean values±1S.D. of triplicate cultures. In single step cultures three low power fields (x40) were counted per well. $2x10^5$ cells were plated per well. In two step cultures the whole dish was scored for colonies. $5x10^5$ cells were plated per well. In two step cultures the same serum was used in both preincubation and agar culture.

The number of colonies that developed was related to the cell concentration. In two step cultures colony formation showed a linear relationship to cell concentration and colonies grew at cell concentrations between $1x10^6$-$3x10^5$ cells/well, with a plating efficiency of about 0.05% (TABLE 1.2) . In single step cultures a plating efficiency ten times that of the two step method was obtained when $2x10^5$ cells were plated per well(FIG 1.2). However at lower cell concentrations the plating efficiency decreased such that no colonies were present when the cell concentration was less than $0.5x10^5$ cells per well ($1.2x10^6$ cells/ml in the agar overlayer). The addition of 1% v/v autologous RBC to underlayers increased the number of colonies and resulted in a less marked fall in plating efficiency with lower cell concentrations. However even in the presence of RBC few or no colonies formed when less than $0.25x10^5$cells ($6x10^5$cells/ml) were plated (TABLE 1.3).The enhancing effect of RBC on colony formation was most marked at low MC concentrations but showed little variation with changes of RBC concentration in the underlayer (between 0.5-2.0%)(TABLE 1.4).At RBC concentrations greater than 2% colonies could only be counted after lysis of the RBC using an acetic acid solution; this resulted in colonies floating free of the agar and clumping thus rendering counts inaccurate.

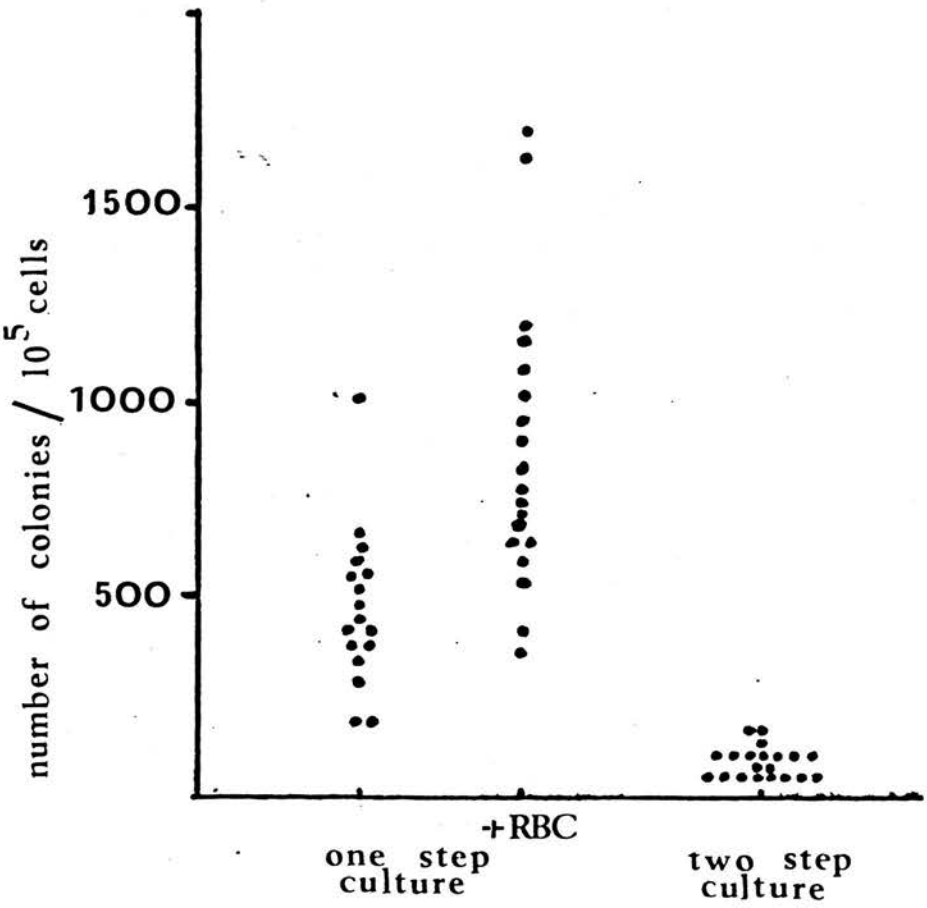

Figure 1.2: Plating efficiencies in one step and two step cultures.

Points are individual values showing the total number of colonies developing from 10^5 blood mononuclear cells from 20 normal individuals.

In one step cultures 2×10^5 cells were cultured in 17 mm diameter multiwell plates using standard culture method or cultures supplemented with 1% v/v autologous RBC. In two step cultures $0.5 - 1.0 \times 10^6$ blood mononuclear cells were cultured in 35 mm diameter petri dishes.

TABLE 1.2

COLONY FORMATION BY BLOOD MC IN TWO STEP CULTURES: EFFECT OF CELL CONCENTRATION ON COLONY FORMATION

No.cells/well($x10^5$)	Total colonies/well	Plating efficiency(%)
10.0	534±73	0.05
5.0	216±24	0.04
2.5	119±35	0.05
1.2	48±14	0.04
0.6	34±14	0.05
0.3	14±8	0.04

Figures are mean values ±1S.D of three experiments using blood MC from 3 different donors.

TABLE 1.3

COLONY FORMATION BY BLOOD MC IN SINGLE STEP CULTURES: EFFECT OF CELL CONCENTRATION ON STANDARD AND RBC SUPPLEMENTED CULTURES

No.cells/well($x10^5$)	Total colonies/well	Plating efficiency(%)
Standard underlayer		
2.0	1147±320	0.57
1.0	300±253	0.30
0.5	23±15	0.05
0.25	0	0
0.125	0	0
RBC underlayer		
2.0	1287±500	0.64
1.0	707±340	0.71
0.5	347±287	0.69
0.25	73±127	0.29
0.125	3±8	0.02

Figures are mean values ±1S.D.of 6 experiments using blood MC from different donors.

TABLE 1.4

ENHANCEMENT OF COLONY FORMATION (SINGLE STEP) BY AUTOLOGOUS RBC

	% enhancement of colony formation			
Underlayer	Standard	0.5%RBC	1.0%RBC	2.0%RBC
$2x10^5$ cells	0 (115±14)	10	29	27
$1x10^5$	0 (14±5)	107	171	150
$0.5x10^5$	0 (6±4)	250	383	250

Figures are the mean of triplicate cultures. Figures in parentheses are the number of colonies in three low power fields on standard underlayers without RBC. %enhancement=[(no. colonies on RBC underlayer - no.colonies on standard underlayer) / no.colonies on standard underlayer] x 100

In single step cultures, colony size, as well as colony number , was also dependent on cell concentration. A progressive decrease in colony size was seen with falling cell concentration. At low cell concentrations although few or no colonies were present, clusters (less than 40 cells) could be seen even when as few as $2.5x10^4$cells were plated (FIG 1.3).

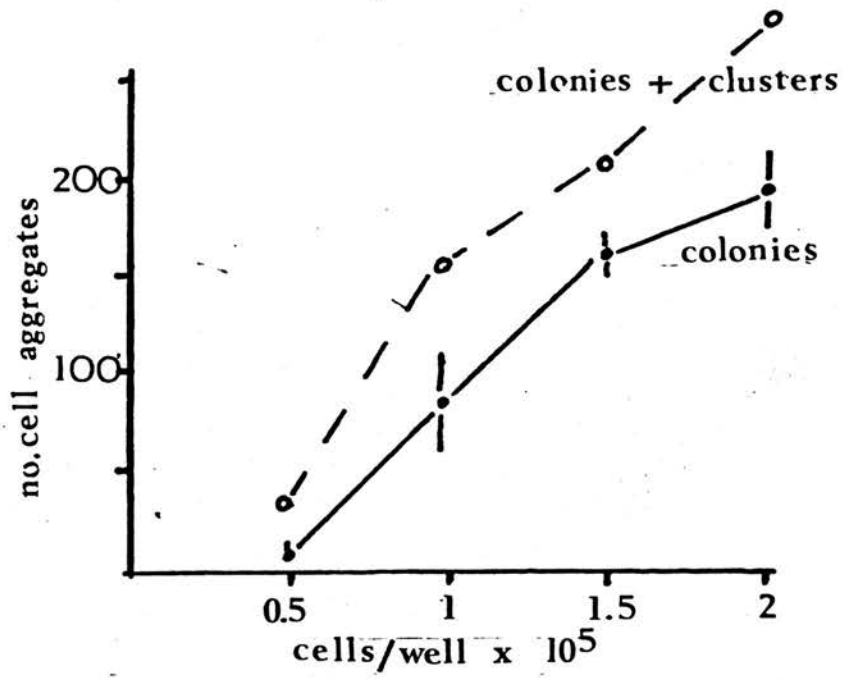

Figure 1.3a: The effect of cell concentration on colony number in one step cultures.

The number of colonies ± S.D. (aggregates >40 cells) and the total number of colonies and clusters (aggregates 4-40 cells) developing at different cell concentration are shown.

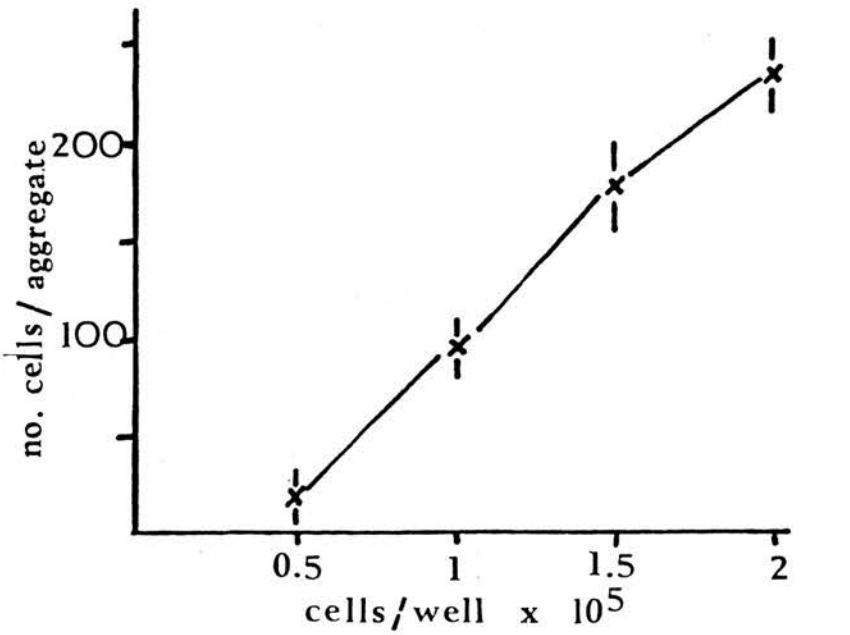

Figure 1.3b: The effect of cell concentration on colony size in one step cultures.

In the same experiment as shown above (Fig. 1.3a), the mean number of cells ± SEM present in cell aggregates (colonies and clusters) developing at different plating concentrations is shown. Aggregate size was determined by counting the number of cells in 50-100 colonies and clusters at each cell concentration.

Phenotypic characteristics of colony cells

In two step cultures colonies formed deep in the agar and were difficult to harvest in sufficient numbers for functional or morphological characterisation. Nevertheless in four experiments about 100 colonies were removed from the agar using a fine pasteur pipette and micromanipulator. After washing and disaggregating the cells it was possible to demonstrate that 44±7% (37-54%) of these cells formed E-rosettes. Insufficient cells were obtained for further studies.

In one step cultures colonies were easily harvested by washing the culture wells with PBS; this led to the detachment of around half the colonies from the agar surface. Between 0.5 and $5x10^6$ cells could be harvested in this way either from a single 90mm plate or from the 24 wells of a multiwell plate.

Cytospin preparations of colonies showed large lymphoblastoid cells, some in mitosis and a small number of small lymphocytes (PLATE 1.3). Cytochemistry showed occasional cells with focal acid phosphatase activity and less than 1% cells with non specific esterase activity . No cells posessed chloroacetate esterase activity. (TABLE 1.5).

TABLE 1.5 IMMUNOLOGICAL AND CYTOCHEMICAL CHARACTERISTICS OF COLONY CELLS

	% positive cells			
	E	SIgM	NSE.	CE
Blood Mononuclear cells (preculture)	67±15 (40-85)	17±11 (5-35)	14±6 (8.5-23)	9±4 (3-13)
Colony cells	83±6 (74.5-91)	2.5±1 (1.5-4.5)	0.2 (0-0.5)	0 (0)

Figures are the mean +1S.D. of six experiments. Figures in parentheses give the range.

TABLE 1.6 IMMUNOLOGICAL TYPING OF COLONY CELL SUSPENSIONS USING MONOCLONAL ANTIBODIES

	% positive cells					
	E	OKT3	OKT4	OKT8	OKT6	HLA-DR
Blood Mononuclear cells	57±13 (43-68)	59±10 (46-69)	48±13 (33-64)	23±8 (16-32)	-	-
Colony cells	96±3 (93-99)	97±2 (93-98)	65±16 (42-78)	39±9 (34-52)	0 (0)	53±22 (22-72)
Thymocytes (1)	89	21	73	81	86	4

Figures are the mean values ± 1S.D. of four experiments.
Values for a thymocyte suspension are included as a control.

Electron microscopy showed cells with rounded or convoluted nuclei, with dispersed chromatin and prominent nucleoli. There was moderate surrounding cytoplasm that contained polyribosomes and mitochondria but few lysosomes and little rough endoplasmic reticulum was seen .

Colony cells therefore possesed morphological and cytochemical characteristics of transformed lymphocytes, with few or no cells showing evidence of plasmacytic, myeloid or monocytic differentiation.

Disaggregated colony cells were further characterised by immunofluorescence and rosetting techniques. Over 80% of colony cells formed rosettes with sheep red blood cells (E-rosettes) (PLATE 1.4). Colony cells did not form rosettes with IgG, IgM, or complement coated ox erythrocytes, nor did they form rosettes with mouse red blood cells. Immunofluorescent staining for surface IgM showed less than 5% positive cells and less than 0.5% cells contained cytoplasmic IgM or kappa or lambda light chains.

Suspensions of colony cells stained with anti T-cell monoclonal antibodies showed over 90% OKT3+ cells, with OKT4+(T-helper) and OKT8+(T- suppressor/cytotoxic) cells present in the same proportions as in blood MC. Colony cells did not stain with OKT6, nor did they show Tdt activity; markers of immature thymic and prethymic T-cells. About half of the colony cells stained with the polyvalent anti HLA-DR antibody DA6.231. (TABLE 1.6).

Immunohistochemical staining of cytospin preparations of non disaggregated colonies gave similar results to immunofluorescent staining of disaggregated cell suspensions. Colonies contained over 90% OKT3 and OKT11 (anti sheep red blood cell receptor) positive cells about half of which were also HLA-DR positive. Variable numbers of cells within individual colonies stained with OKT4 and OKT8, the appearances suggesting that colonies contained a mixture of OKT4 and OKT8 positive cells. Many of the colonies contained a central OKM1, MO2, Ia positive macrophage. Colonies did not stain with the antibodies B1(anti-B cell) or DA6.127 (anti-IgM) (PLATES 1.5-1.8).

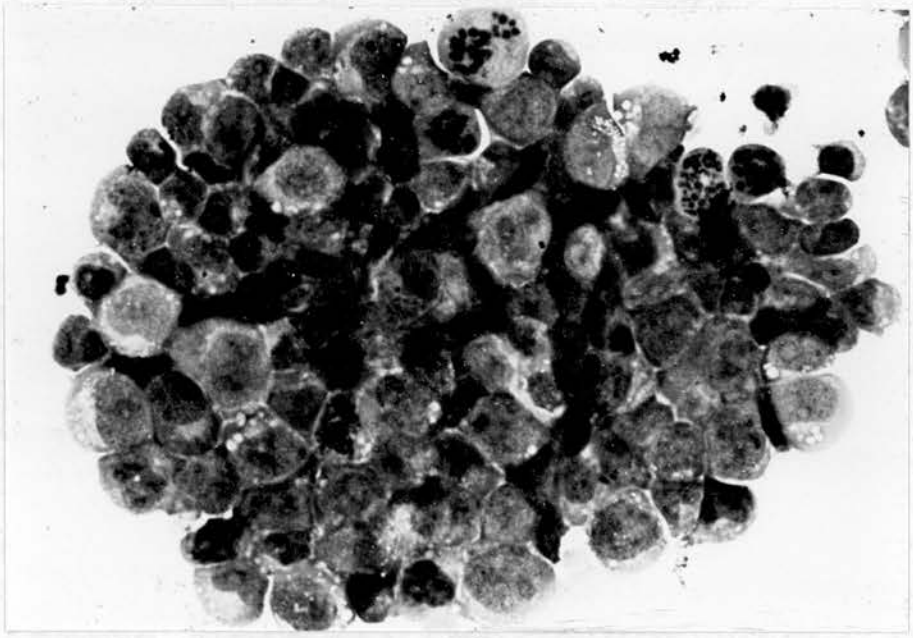

PLATE 1.3 T-LYMPHOCYTE COLONY
Cytospin preparation
May-Grunwald-Giemsa (original magnification x250)

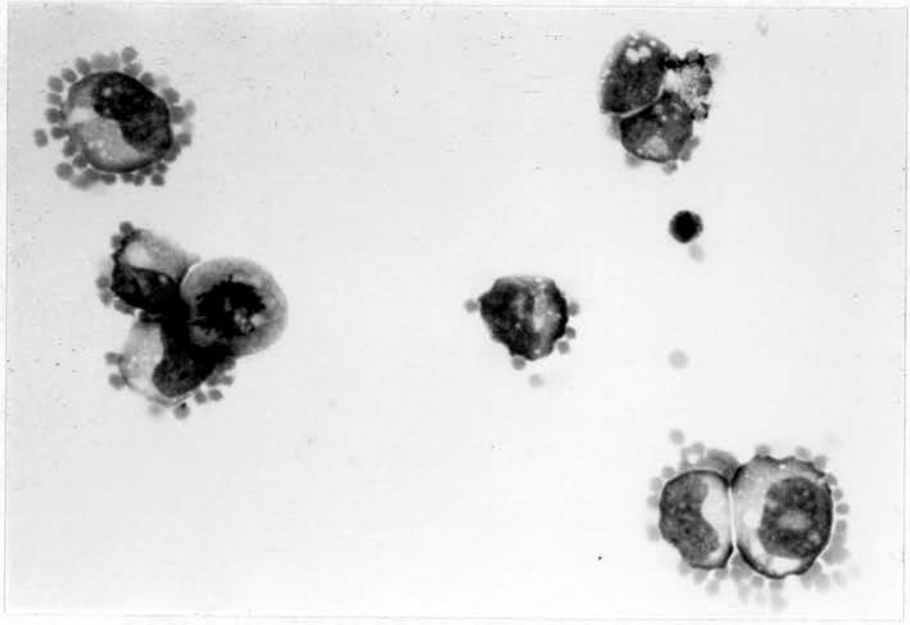

PLATE 1.4 T-COLONY CELLS; E-ROSETTES
Cytospin preparation
May-Grunwald-Giemsa (original magnification x400)

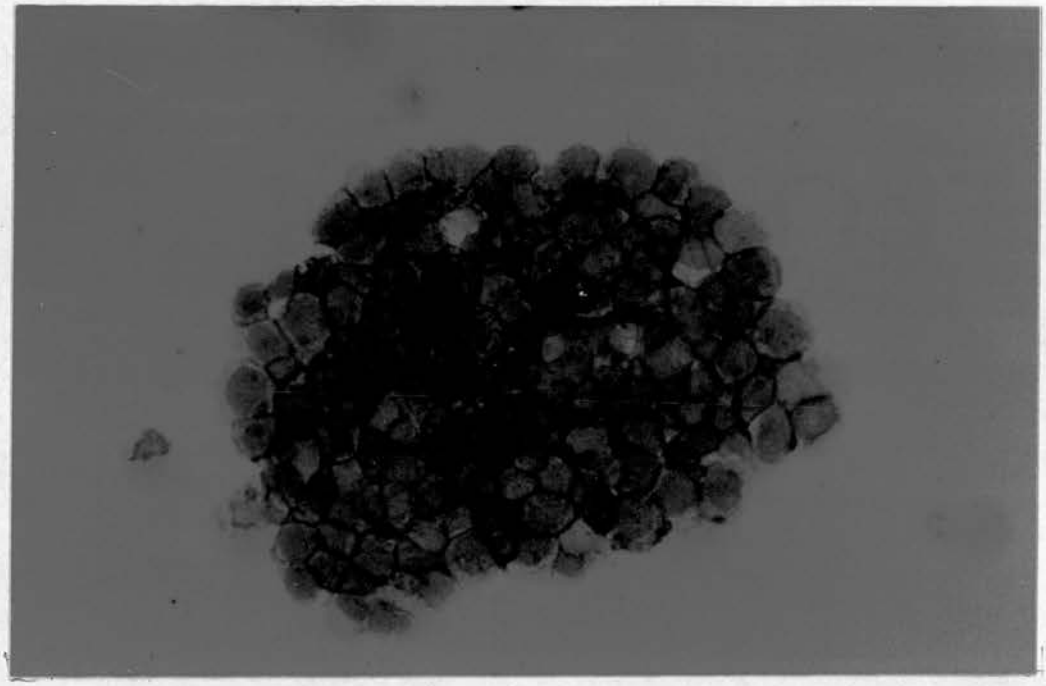

PLATE 1.5: T cell colony stained with OKT11
(original magnification x 250)

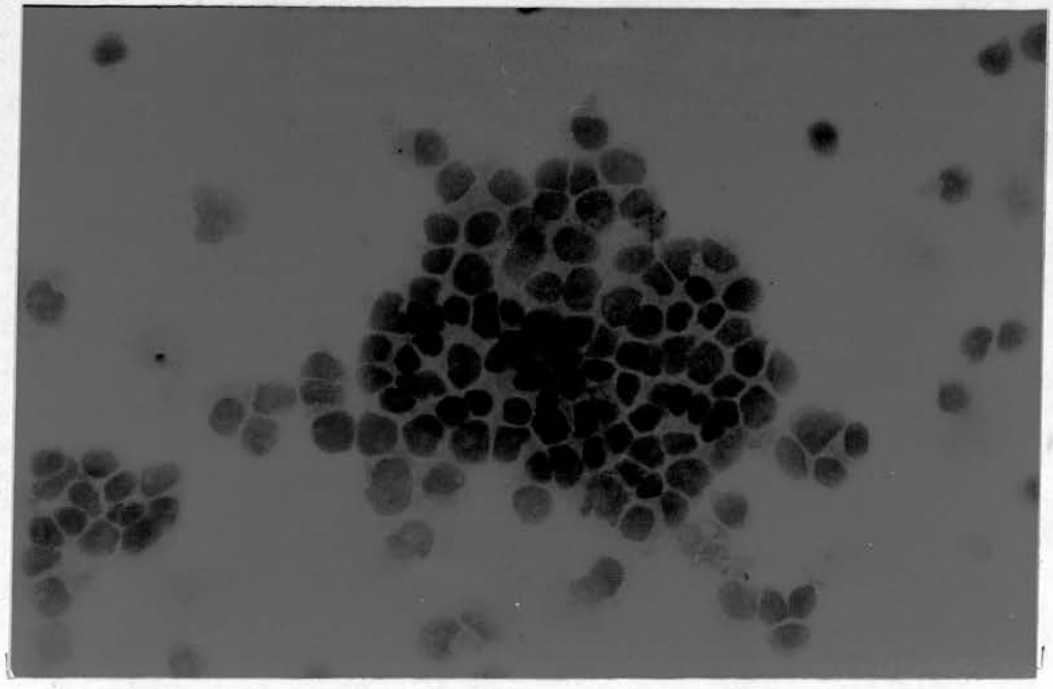

PLATE 1.6: T cell colony stained with M02
(original magnification x 250)

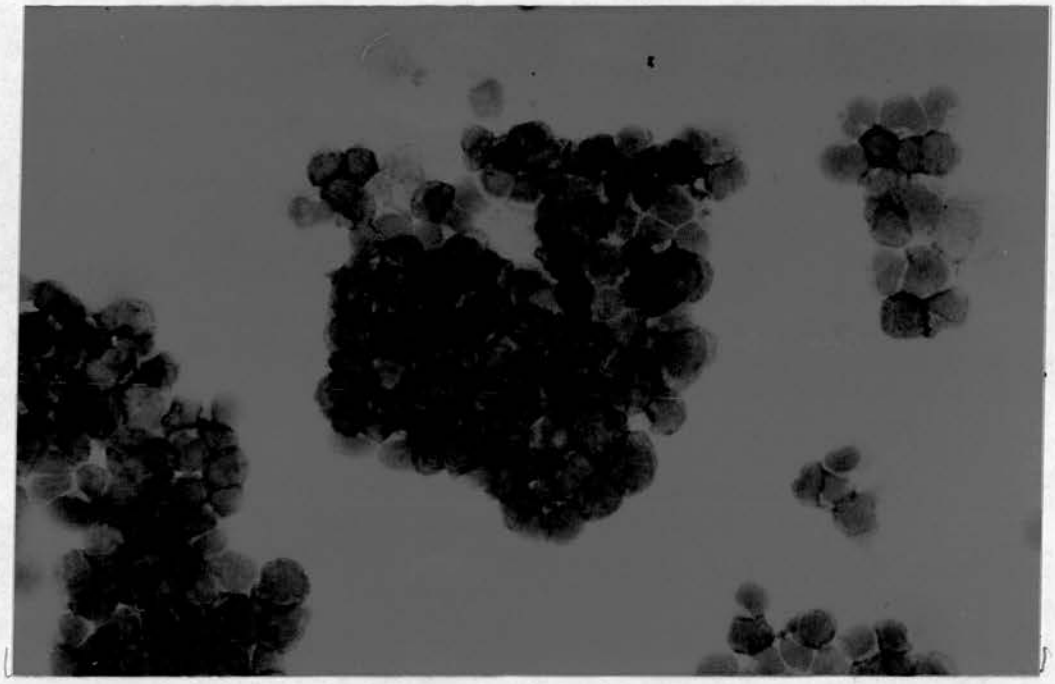

PLATE 1.7: T cell colony stained with OKT4
(original magnification x 250)

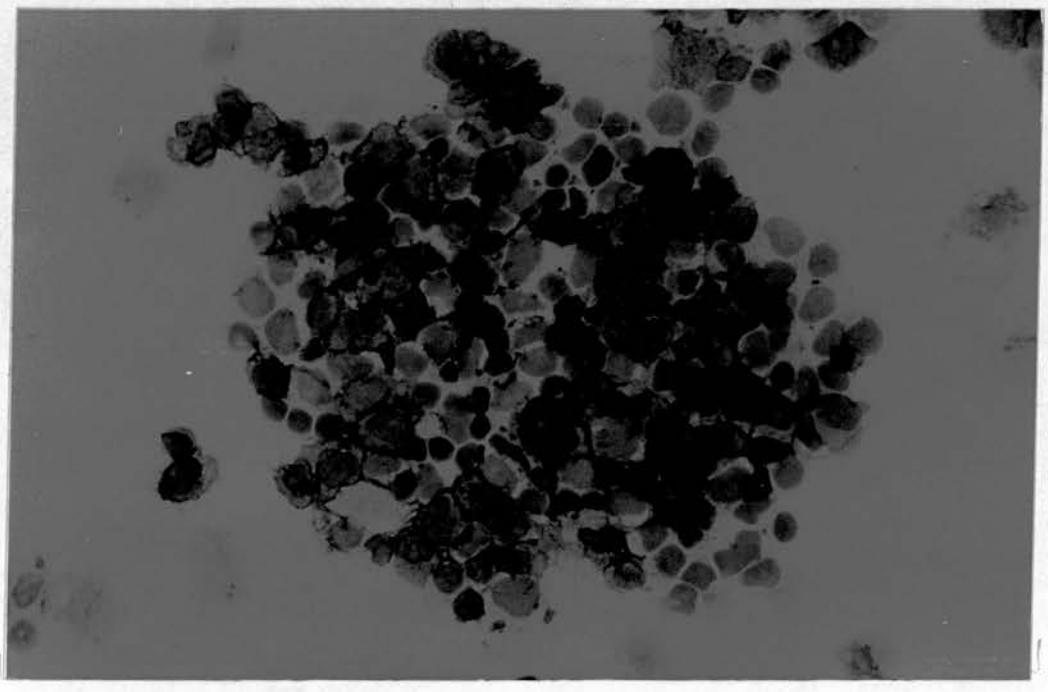

PLATE 1.8: T cell colony stained with OKT8
(original magnification x 250)

DISCUSSION

These studies confirm previous observations that human blood MC can be induced to form T cell colonies in agar when stimulated with PHA . Colony formation could be induced either by plating cells directly into agar (single step cultures) or by preincubating cells in suspension with PHA and then plating into agar (two step cultures). The plating efficiencies observed (0.05% for two step and 0.5% for single step) are similar to those reported by previous workers using the same techniques (Rosenszajn et al 1975, Riou et al 1976).The increase in plating efficiency obtained by using human serum (or plasma) rather than foetal calf serum and by incorporating red blood cells into cultures is consistent with previous observations (Gerassi and Sachs 1976, Claesson et al 1977a, Woods and Lowenthal 1981).

It has been suggested that the low plating efficiency of two step cultures may be attributable to colonies developing from aggregates of cells rather than single cells (Claesson et al 1977a). In the present study it was found to be difficult to completely disaggregate cells following preincubation with PHA and when cells were plated into agar small clusters of cells were often present at the beginning of culture. This problem of cell clumping was not encountered when freshly isolated blood MC were plated directly into agar in the single step cultures. However even in the single step culture system colony formation was dependent on plating cells at a

relatively high cell concentration in the thin agar overlayer, suggesting that some degree of cell crowding and contact might be necessary to allow colony formation. Others have also observed a similar requirement for a minimum number of cells to be plated in order to obtain colony formation (Riou et al 1976, Claesson et al 1977a, Shen et al 1977, Gerassi and Sachs 1978, Goube deLaforest et al 1978,1979a).

These findings suggest that colonies may not be clonal in origin but originate from clusters of cells, particularly in the two step cultures. Studies by Singer et al (1981) using lymphocytes from individuals heterozygous for G6PD, and carrying GdA and GdB isoenzyme markers have shown that colonies grown from these individuals contain a mixture of the two enzymes in over 90% of two step colonies and in 70% of one step colonies. Mathematical analysis of these results indicated that in two step cultures colonies on average developed from 10 cell clusters and in single step cultures from 2-3 cell clusters (Singer et al 1981). These findings conflict with the observations of Gerassi and Sachs (1978) who, using mixtures of lymphocytes from normal and G6PD deficient donors, could only detect 20% mixed colonies; their finding may however be the result of a failure of colonies to develop from clusters containing allogeneic cells (Farcet et al 1980). Rosenszajn et al (1975) claimed to observe colony development from single cells in culture and even Singer et al (1981) noted that, by morphological examination of two step cultures, most colonies apparently originated from clusters of

no more than 2- 4 cells. It therefore seems probable that differences in the plating efficiency between one step and two step cultures are not solely attributable to cell clumping and although this plays some part other factors may be involved: e.g the higher plating efficiency of two step cultures may result from a proliferation requirement for direct cell- cell interactions or interactions dependent on adequate local levels of growth factor production, both of which are more likely to occur at the higher cell plating concentrations used in the one step method. The fall in plating efficiency that occurs with falling cell concentration in one step cultures is consistent with this interpretation and is supported by subsequent observations of cell interaction requirements in colony formation (see Chapter 2).

In order to confirm that the colonies grown in these studies were of T- cell type, colony cells were harvested from agar for immunological and cytological characterisation. A problem with two step cultures, was that the majority of colonies formed deeply within the agar overlayer with only a few small colonies on the agar surface; these correspond to the type 1 (deep) and type 2 (superficial) colonies described by other groups (Rosenszajn et al 1975, Fibach et al 1976). Because of this it was difficult to harvest colonies free of agar and only small numbers of cells ($<1x10^5$) could be obtained from the culture dishes. Colony cells from these cultures showed 40-50% E- rosetting cells. This figure is similar to that reported by others (Rosenszajn et al 1975,

Fibach et al 1976, Claesson et al 1977a). The low value of E-rosettes has been attributed to agar adhering to cells or persisting in the culture medium, rather than to colonies containing a mixture of T and non T cells (Claesson et al 1977a, Galbraith et al 1977).

In one step cultures colonies were readily harvested in large numbers from culture dishes. Colony cells showed typical cytological and cytochemical characteristics of T- lymphocytes undergoing blast transformation. Over 90% of colony cells showed immunologic characteristics of mature T- cells i.e they formed rosettes with sheep red cells (E- rosettes) and reacted with the T-cell specific antibodies OKT3 (pan T), OKT4 (helper- inducer subset) and OKT8 (suppressor cytotoxic subset). Colony cells did not react with OKT6 (immature T cell, thymocyte) nor did they show Tdt activity characteristic of immature T cells (Reinherz et al 1980a). In the present study 40-60% of colony cells expressed HLA-DR antigens characteristic of T cell activation. Gelfand et al (1981) have reported similar results.

Immunofluorescent and immunohistochemical staining of intact colonies (rather than disaggregated single cells) gave similar results. Interestingly most colonies appeared to contain a mixture of OKT4 and OKT8 positive cells and a central HLA-DR+, OKM1/MO2+ cell i.e. a macrophage. These findings are consistent with colonies developing from several T- cells clustered around a macrophage rather than a single

cell, although it remains possible that colonies develop from a precursor that differentiates into OKT4+ and OKT8+ cells (Spitzer et al 1982, Claesson et al 1983). This is discussed further in chapter 2.

In contrast to the results of others no Fc or C3 receptors could be demonstrated on colony cells (Claesson et al 1977a, Dao et al 1978). This discrepancy is probably the result of differences in the methodology used for detecting EA (Fc) and EAC (C3) rosetting cells; these groups used antibody and complement coated sheep erythrocytes, rather than ox red cells as used here. Coated sheep red blood cells may however bind non specifically to T cells (by E-receptor) even when coated with antibody (Palutke et al 1977).

In the present study a small number of cells (<5%) with surface Ig were found in colony cell suspensions. A similar finding was reported by Goube de Laforest et al (1979 a). It is probable that these cells include both cells with non specifically bound antibody (e.g. antibody bound to T cells or macrophages via Fc receptor) and individual blood B-cells persisting in cultures without proliferation . Cytospin preparations of intact colonies showed no colonies with plasmacytoid features or expressing B-cell antigens (B1 and cytoplasmic Ig) and the culture conditions are not those which would be expected to support B- cell colony formation (Radnay et al 1979, Bobak and Whisler 1980).

These studies show that although both one and two step culture methods can be used for PHA induced T-cell colony formation, the one step method appears to offer several advantages. These advantages included the convenience of a single step procedure, a higher plating efficiency and a simple method for harvesting colony cells for characterisation and for possible future functional studies. For these reasons single step cultures were chosen for subsequent investigations.

CHAPTER 2

CELL INTERACTIONS AND COLONY STIMULATING FACTORS IN COLONY FORMATION

SUMMARY

In this chapter studies of cell interactions in colony formation are described. These studies included investigation of the role of monocyte and lymphocyte derived growth factors. Monocyte (MoCM) and Lymphocyte conditioned media (LyCM) (with interleukin 1 and interleukin 2 activity respectively) were both found to enhance colony formation by blood mononuclear cells and purified blood T cells in primary culture. Maintenance of proliferation of colony cells in secondary culture was however wholly dependent on a factor present in LyCM (probably interleukin 2).

Using a mathematical approach (kinetic analysis) to analyse cell interactions, it was determined that at least three interacting cells are involved in PHA induced, primary T cell colony formation by blood mononuclear cells. Further analysis showed that the requirements for cell interactions in primary colony formation could be reduced or eliminated by the inclusion of MoCM and LyCM into cultures. These findings are discussed in relationship to the findings of other authors and to current concepts of interleukin mediated regulation of T lymphocyte proliferation.

It can be postulated that binding of lectin to T-cells directly triggers their entry into and their passage through the cell cycle. However experimental observations of both colony formation and proliferation in liquid culture have indicated that this is unlikely as proliferation is dependent upon interactions between mitogen, accessory cells and cells with a proliferative potential (Andersson et al 1979, Couthino et al 1979, Rosenszajn et al 1981). Both macrophages and lymphocytes have been implicated as accessory cells.

Claesson et al (1977a,b) demonstrated that there was a 60-85% reduction in colony formation by blood MCs depleted of adherent cells. Colony formation was restored to normal levels by culturing MC either on an adherent cell feeder layer or by adding conditioned medium from adherent cell cultures. Similar findings were reported by Rosenszajn et al (1978) who showed that depletion of macrophages from blood MC reduced colony formation and that this could be restored by the addition of macrophages to the cultures. Earlier studies by Rosenszajn's group had failed to demonstrate any colony stimulating activity in adherent cell culture supernatants (Zeevi et al 1977) but later studies showed that medium conditioned by exposure to adherent cells contained a high molecular weight colony promoting factor (LCPF) whose activity was demonstrable following dialysis and removal of a low molecular weight inhibitory factor (LCIF) (Rosenszajn et al

1978, 1980, 1981).

Evidence that other accessory cells, in addition to the macrophage, might play a role in colony formation was provided by studies of Goube deLaforest et al (1979a, 1979b) and Klein et al (1982). In contrast to other groups they were unable to show colony stimulating activity in monocyte culture supernatants (Klein et al 1982), but they demonstrated that in primary culture colony formation by purified T- cells, and to a lesser extent unfractionated mononuclear blood cells, was enhanced by conditioned medium from PHA stimulated lymphocytes (Klein et al 1982). This PHA conditioned medium was essential for colony formation in secondary cultures of T-colony forming cells harvested from primary colonies and replated in secondary cultures (Goube deLaforest et al 1979a). From mathematical analysis of the number of colonies produced at different cell concentrations in primary cultures they deduced that colony formation was dependent on interactions between at least three cells (Goube deLaforest et al 1979b), and suggested that colony formation by T- cells was dependent on interactions between a colony forming cell and two accessory cells; one accessory cell being a lymphocyte and the other a monocyte (Klein et al 1982). This is consistent with numerous observations by other groups that both monocytes (Claesson et al 1977b, Rosenszajn et al 1978) and PHA stimulated lymphocytes produce colony stimulating factors that enhance colony formation in primary cultures (Zeevi et al 1977, Rosenszajn et al 1978, Gerassi and Sachs 1978). The failure of

some groups (Klein et al 1982) to demonstrate colony stimulating activity by monocytes may be attributable to the contamination of monocyte stimulatory factors by inhibitory factors, such as prostaglandins, also present in conditioned medium (Bockman and Rothschild 1979).

Data on accessory cells and their products is summarised in table 2.A.. Although this table lists the various colony stimulating factors under the names given by the various groups, these factors were not fully characterised at the time of their first description and do not necessarily represent separate entities. It is apparent however that these colony stimulating factors fall into two main groups; a factor produced by adherent cells (macrophages/monocytes) and a factor produced by PHA stimulation of lymphocytes.

The three cell model for T-cell colony formation , described above, shows marked similarities to models for interleukin regulated proliferation of lymphocytes , which include a cell capable of clonal proliferation and two accessory cells; one a lymphocyte producing interleukin 2 (IL-2) and the other a monocyte producing interleukin 1 (IL-1) (Smith et al 1980, Palacios 1982). The main biological and biochemical characteristics of IL- 1 and IL-2 are summarised in tables 2.B,2.C..

TABLE 2A FACTORS MODULATING T-LYMPHOCYTE COLONY FORMATION

Factor	source	biochemistry	Reference
Lymphocyte colony enhancing factor LCEF	T cells+PHA blood MC+PHA	90-110K protein	Zeevi et al 1977 Rosenszajn et al 1978, 1980, 1981
Lymphocyte colony promoting factor LCPF	spleen adherent cells, peritoneal exudate cells	10-30K protein	"
Lymphocyte colony inhibitory factor LCIF	spleen and blood adherent cells	<1K (PGE1,PGE2)	" Bockman and Rothschild 1979
T colony inducer TCI	T-cells+PHA blood MC+PHA adherent cells	not known	Gerassi and Sachs 1978
Adherent cell factor	blood adherent cells	12-15K 100K protein	Claesson et al 1977b
Colony promoting activity CPA	blood MC+PHA	not known	Goube deLaForest et al 1979a,b

The factors listed in this table are named as described by the authors. Several of these factors are probably biochemically identical.(see text)

TABLE 2B PRODUCTION AND BIOCHEMICAL CHARACTERISITICS OF IL-1 AND IL-2

	IL-1	IL-2
Production:		
	monocytes + LPS/endotoxin	T-lymphocytes (OKT4+,Lyt1+)
	monocytic and myelomonocytic	+ mitogens (PHA,ConA,TPA)
	leukaemia cells	+ antigen
	murine macrophage cell line	Murine T-lymphoma lines
	P388D1	Human T-lymphoma line
	Murine myelomonocytic cell line	(Jurkhat FHCRC)
	WEHI3	some T-cell hybridomas
Biochemistry:		
Human	15K protein (gel filtration)	15K protein
	PI 6.5-7.5 (iso-electric focussing)	6-6.5
Murine	15K protein	30K protein
	PI 4.5-5.5	4.3-4.9

for references see text

TABLE 2C BIOLOGICAL ACTIVITIES OF IL-1 AND IL-2

Activity	IL-1	IL2
Direct mitogenic effect on thymocytes	+(high concentration)	-
Enhancement of thymocyte and T-lymphocyte mitogenic responses to PHA and ConA	+	+
Enhancement of IL-2 production by T-cells	+	-
Maintenance of long term proliferation of antigen/mitogen activated T-cells (Tcytotoxic, suppressor and some helper T-cells)	-	+

for references see text

IL-1 was first described by Gery et al (1971) as a factor present in leucocyte culture supernatants that enhanced proliferation of murine thymocytes. Subsequent studies have shown that IL-1 is produced by human and murine adherent cells (Gery and Waksman 1972), murine peritoneal exudate cells (Calderon and Unanue 1975) and human blood monocytes (Blyden and Handschumacher 1977) as well as by murine (Mizel et al 1978) and human monocytic cell lines and myelomonocytic leukaemias (Lachman et al 1978). The production and release of IL-1 from monocytes is stimulated by phagocytosis (Unanue et al 1976), endotoxin (Gery et al 1972, Lachman et al 1977) and also by products derived from activated T-cells (Mizel et al 1978). IL-1 enhances the response of murine and human thymocytes and T-cells to mitogens, but does not support long term proliferation of T- cells in vitro (Oppenheim et al 1980, Mizel 1982).

IL-2 has been defined as a lymphokine that can maintain long term proliferation of normal T-cells in vitro (Morgan et al 1976, Aarden et al 1979). IL-2 is produced by T-lymphocytes, with a T-helper phenotype following stimulation with mitogens, and also by stimulation with specific antigen (Reinherz et al 1980b, Palacios 1982). Recently IL-2 production by murine and human T-cell lines has also been demonstrated (Mochizuki et al 1980, Gillis and Watson 1980). The production of IL-2 by normal T-lymphocytes is dependent on the presence of both T- cells and macrophages or IL-1 (Larsson and Couthino 1979, Couthino et al 1979, Larsson et al 1980,

Smith 1980, Smith et al 1980).

IL-2 has been shown to bind to a specific IL-2 receptor that appears following activation of IL-2 responsive cells (Robb 1982). Proliferation of IL-2 responsive cells can be maintained, probably indefinitely, as long as IL-2 is added to cultures (Ruscetti et al 1976, Gillis et al 1978a).

At present the relationship of the interleukins to the factors that regulate T- cell colony formation is uncertain. Both in terms of production and biochemical characteristics IL- 1 and IL- 2 show similarity to the described colony stimulating factors (see tables 2A,B,C). Lymphocyte colony enhancing factor (LCEF) prepared from PHA stimulated cells has a mol.wt 90- 110K (Rosenszajn et al 1980) that corresponds to high molecular weight IL-2 activity described by Oppenheim et al (1980). This high mol.wt activity may represent complexed low mol.wt units as Gillis et al (1980) have shown that following dialysis against 0.5M NaCl such high mol.wt fractions in PHA MC conditioned media when rechromatographed give a peak of IL-2 activity at 15K. Lymphocyte colony promoting factor (Rosenszajn et al 1980) and the adherent cell factor described by Claesson et al (1977b) are biochemically similar to IL-1 which is a protein with a mol.wt of 15K (Lachman et al 1977, Blyden and Handschumacher 1977). Although the classically defined interleukins 1 and 2 may enhance colony formation (Claesson et al 1977b, Mossalayi et al 1982), there is evidence that colony formation may also be

regulated by other less well defined factors (Spitzer et al 1982).

The aim of the studies described in this chapter was to determine the cell interaction requirements for colony formation and in particular the role of monocyte and lymphocyte derived growth factors in regulating T-cell colony formation. This was done by two techniques:

Firstly I investigated the effect of conditioned media with IL-1 and IL- 2 activity on colony formation. The ability of conditioned media to stimulate colony formation in primary cultures containing either unfractionated mononuclear cells or purified T-cells was compared and the ability of such media to maintain proliferation of colony cells in secondary cultures was measured.

Secondly I used a mathematical model, that allows kinetic analysis of colony formation, to deduce the number of interacting cells (Goube deLaforest et al 1979a) and to analyse the ability of conditioned media to substitute for cell interactions.

MATERIALS AND METHODS

PREPARATION OF CELL SUSPENSIONS

Blood MC were isolated as described in chapter 1.

Spleen MC were obtained from a patient who had undergone splenectomy for congenital spherocytosis. Spleen MC were isolated by scraping the cut surface of spleen with a scalpel into heparinised (10units/ml) TC199. This suspension was then passed through a fine wire mesh and then layered onto Ficoll-Hypaque. After centrifugation cells from the medium-Ficoll interface were washed twice and resuspended at the required cell concentration in RPMI1640. The viability of the spleen MC was greater than 95%; 88% of these cells formed E-rosettes. Spleen MC were used to prepare spleen lymphocyte- conditioned medium (see below).

Thymocytes were obtained from thymuses removed from children undergoing cardiac surgery . The cut surface of the thymus was scraped into heparinised TC199 and the resulting thymocyte suspension was then washed twice and resuspended in RPMI 1640 at the required cell concentration.The viability of the thymocyte suspensions used was greater than 90%.

Tonsil and lymph node cell suspensions were obtained from surgical specimens, shown to be histologically normal, using the same method as described for thymus.

Preparation of T cell enriched and depleted MC populations:- T cell enriched and depleted cell populations were prepared by rosetting MC with neuraminidase treated sheep red blood cells (n-SRBC), followed by centrifugation over Ficoll- Hypaque . MC were incubated with n-SRBC at a ratio of 1:40 in RPMI 1640 with 5%FCS for 5minutes at 37°C., centrifuged for 5 minutes to pellet and then incubated on ice for 45 minutes. The cell pellet was then gently resuspended and layered onto Ficoll- Hypaque and centrifuged for 20 minutes at 1200g.. Cells at the Ficoll interface (T- depleted) were removed and washed twice . Cells in the pellet were resuspended in medium at 4°C. and again layered onto Ficoll and centrifuged. The supernatant was removed and the cell pellet resuspended in a small volume of medium. The red cells were lysed by adding hypotonic medium (1 volume distilled water: 9 volumes of RPMI 1640). The remaining mononuclear cells were then washed twice and resuspended at the required cell concentration. The T cell enriched fraction contained more than 90% E positive cells and the T depleted fraction less than 5% E positive cells.

Depletion of T cells and Monocytes from cell suspensions by treatment with monoclonal antibodies and complement

Aliquots of $5x10^6$ cells were pelleted in round bottomed glass tubes (12x7mm) and the supernatants removed. Twenty microlitres of the appropriate antibody (OKT3, OKT4, OKT6, OKT8 or OKM1) was then added to the tube (diluted 1/2 in PBS-

2%HSA) and the tubes incubated at room temperature for 45 minutes. Following this the cells were washed once in PBS-2%HSA and resuspended in 0.1ml PBS-2%HSA, and 0.1ml of fresh AB,Rh-positive human serum was added. Cells were then incubated for 45 minutes at 37°C, washed twice in RPMI 1640 and resuspended at the required concentration.

COLONY FORMATION

Primary colony formation

Colony formation by freshly isolated MC was carried out using a single step culture method as described in chapter 1 , but with some modifications. In addition to the standard culture method already described cultures were prepared in which conditioned media were added to the underlayers. Conditioned media used to supplement the cultures were prepared as described below. Lymphocyte conditioned medium (LyCM) and monocyte conditioned medium (MoCM) were usually incorporated in underlayers at 25% v/v and 15% v/v concentrations , either alone or in combination.

In some experiments colony formation was assessed in the presence of adherent cell underlayers. These were prepared by adding freshly isolated MC to culture wells (0.1ml of cell suspension at $10x10^6$cells/ml in RPMI 1640 with 20% human serum) and incubating for 4 hours at 37°C. Following this the wells were washed at least three times by aspirating the supernatant and adding fresh medium, thus leaving a relatively

pure population of adherent cells attached to the well bottom. After this the standard culture procedure was followed, the culture underlayers being added to wells containing adherent cells.

Secondary colony formation

Colony cells (T-CFC) were harvested from primary cultures as described in chapter 1. After washing and disaggregation these were used to measure colony formation in secondary cultures. The culture procedure was identical to that described for primary cultures with the exception that cultures were prepared both with and without PHA in the underlayer. Human serum (pooled heat inactivated human serum) was used in place of autologous plasma.

Colonies were scored as described in chapter 1. Primary cultures were scored after 6-7 days incubation and secondary colonies after 5-6 days incubation. In some experiments colony size was measured . This was done either by photographing individual wells and counting the number of cells in colonies in photographic prints or by directly examining colonies at x100 magnification using an inverted microscope.

PREPARATION OF CONDITIONED MEDIA

Preparation of conditioned medium with interleukin-1 activity (Monocyte conditioned medium - MoCM)

MoCM was prepared as described by Lachman et al (1978), using human monocytic leukaemia cells obtained from a patient with acute monocytic leukaemia . Leukaemic cells were isolated from blood by centrifugation over Ficoll-Hypaque and stored in 20% DMSO in foetal calf serum in liquid nitrogen. The leukaemic cells showed morphology typical of Schilling type monocytic leukaemia, and were greater than 90% non specific (alpha naphthyl acetate) esterase positive , were chloroacetate esterase negative. These cells reacted strongly with anti-monocyte serum (Stuart et al 1976) and contained only 8% E-rosette positive cells.

To prepare MoCM leukaemic cells were taken from storage in liquid nitrogen, thawed, washed and resuspended in RPMI 1640 containing 5% human serum and 10-5M. 2-mercaptoethanol (Sigma) at 0.5×10^6 /ml. This suspension was incubated for 24 hours at 37°C in 10% CO2 in air, in Roux flasks. The supernatants were collected after centrifugation at 800g for 20 minutes , aliquoted, and stored at -20°C until used.

Preparation of conditioned medium with interleukin-2 activity [Lymphocyte conditioned medium - LyCM]

LyCM was prepared by a method modified from Morgan et al

(1976). Spleen MC were obtained as described above. These were suspended at $1x10^6$ cells/ml in RPMI 1640 containing 1% human serum and 1% PHA. This suspension was incubated at 37°C in a humidified 5% CO2 in air atmosphere , in 25ml Falcon flasks , for 48 hours. The supernatants were collected after centrifugation at 500g for 10 minutes , aliquoted and stored at -20°C. Unless otherwise stated this spleen LyCM was used in the experiments described in this chapter. Other batches of Ly-CM were prepared by incubating MC from blood, disaggregated thymus or other sources under the same conditions. When these were used instead of spleen LyCM this is stated in the text.

BIO-ASSAYS DEMONSTRATING INTERLEUKIN ACTIVITY

Interleukin-1 (IL-1 assay)

IL-1 activity in conditioned medium was detected by demonstrating a direct mitogenic effect on thymocytes and by demonstrating enhancement of PHA induced thymocyte mitogenesis (Maizel et al 1981). Human thymocytes (either freshly isolated or from storage in liquid nitrogen) were incubated at $1x10^6$ cells/ml in RPMI 1640 containing 10% human serum or foetal calf serum, together with the test CM at concentrations between 0-50%. All cultures were performed in triplicate both with and without 0.5% PHA. Cultures were either carried out in Linbro multiwell dishes (24x17mm diameter wells) or in Nunc multiwell plates (96x5mm diameter wells). In both cases 0.2ml of suspension ($2x10^5$cells) was added to the wells (Maizel et

al 1981). Cultures were incubated for 72 hours , at 37°C , in a humidified 5% CO2 in air atmosphere. Proliferation was assessed by tritiated thymidine (3H TdR) incorporation as described below.

In order to demonstrate that MoCM enhanced IL-2 production experiments were also performed in which lymphocytes (thymocytes, blood MC or purified T-cells) were incubated with PHA with or without 15% MoCM, under the conditions described above for the preparation of LyCM. Supernatants from these cultures were tested for IL-2 production.

Interleukin-2 (IL-2 assay)

IL-2 activity was detected using techniques similar to those described by Gillis et al (1978b). Target cells for testing IL-2 activity were T colony cells (T-CFCs) harvested from primary cultures after 6- 7days incubation. These cells respond poorly or not at all to PHA but proliferate if Ly-CM is present . They therefore behave in an identical manner to conventional target cells used in IL-2 assays i.e. T cells maintained in suspension culture with LyCM for two or more weeks (Inouye et al 1980, see also results). T- CFCs were suspended at $1x10^5$ cells/ml in RPMI 1640 containing 10% human serum and CM at concentrations between 0-50%. Cultures were incubated for 96 hours under the same conditions as described above for thymocytes in the IL-1 assay. Cell proliferation was assesed by 3H TdR uptake.

Measurement of 3H TdR incorporation

Tritiated thymidine (methyl 3H thymidine 40-60 Ci/MMol, Radiochemical centre, Amersham) was added to cultures 4 hours prior to termination to give a final concentration of 1.0 microCi/ml. At the end of the culture period ice cold PBS was added to the wells and samples from the wells were aspirated and pipetted either onto Whatman GF/C filters mounted in a multiple sampling harvester (3025 sampling manifold , Millipore) or aspirated automatically onto filters using an automatic harvester (Multimash cell harvester, Dynatech Laboratories Ltd.). The cells were washed with 0.85% NaCl and then with 5% TCA. The filters were then dried and placed in 4.5ml of scintillant (Ne260 micellar scintillator, Nuclear Enterprises Ltd. or Scintillator 299, Packard), in mini-vials. 3H-TdR incorporation into acid precipitable material was measured in a Tracor Analytic Delta 300, 6891 liquid scintillation system, with an efficiency for 3H of approximately 50%.

KINETIC ANALYSIS OF COLONY FORMATION

This type of mathematical analysis has previously been used to investigate cell interactions in mitogen induced lymphocyte activation and proliferation in both liquid cultures (Kondracki et al 1977, Tse et al 1980) and in agar cultures (Goube deLaForest et al 1978, 1979b, Kondracki

1979). The basic principle is to consider cells as kinetic particles. If a cellular response, such as colony formation, is dependent upon cell interactions between a number (n) of different cell types, then the probability of cell interaction (and the subsequently detected response) depends on the concentration of each of the cell types present. This can be represented mathematically by an equation similar to that for the law of mass action (Tse et al 1980):

$$\text{Response}(R) \propto [\text{cell-1}] \times [\text{cell-2}] \times \ldots\ldots [\text{cell-n}]$$

$$R = C \times a1[X] \times a2[X] \times \ldots\ldots\ldots an[X]$$

Where R is the response, C is the constant of proportionality,[X] is the total cell concentration and ai = [ai]/[X], i=1 to n.

$$\text{Hence } R = C\,(a1 \times a2 \times \ldots . an)\,[X]^n$$

By taking the logarithm of the equation:

$$\log R = n \log [X] + K$$

$$\text{Where } K = \log C + (\log a1 + \log a2 + \ldots \log an)$$

Hence a plot of log R against log [X] should give a straight line, with a slope n, where n represents the number of cells interacting to give the response R.(Tse et al 1980).

In this study the response measured was colony formation obtained by plating cells at different cell concentrations. The following equation was used to calculate the requirement for cell interactions:

$$\log n = b \log N + a \quad \text{(derived from } \log R = n \log[X] + K\text{)}$$

where n = number of colonies, N = number of cells plated, b = number of interacting cells. The value of b (slope of the line logn/logN) was calculated by simple regression analysis.

By culturing MC on standard underlayers with PHA alone and also on underlayers containing conditioned media, it is possible to compare regression coefficients (b) under different conditions and hence to determine whether CM can substitute for cellular interactions.

RESULTS

1. ENHANCEMENT OF T-CELL COLONY FORMATION BY MONOCYTE AND LYMPHOCYTE CONDITIONED MEDIA

1A. PREPARATION AND BIOASSAY OF CONDITIONED MEDIA

This section describes the T cell stimulatory activity of monocyte and lymphocyte conditioned media (MoCM, LyCM). IL-1 activity was detected by demonstrating enhancement of PHA induced thymocyte mitogenesis and also by enhancement of IL-2 production by thymocytes and T lymphocytes. IL-2 activity was detected by demonstrating the ability of conditioned media to maintain the proliferation of cultured T cells after loss of responsiveness to PHA and IL-1.

MoCM enhanced PHA induced thymocyte mitogenesis and also showed a small but consistent direct mitogenic effect on thymocytes (TABLE 2.1A). Optimal stimulation of thymocytes occured when MoCM supplemented the culture medium at 12% v/v concentration, with less marked stimulation at higher or lower concentrations (TABLE 2.2). MoCM showed little or no stimulatory activity when tested on cultured T-cells (TABLE 2.1B and see next paragraph), but did enhance the mitogenic response of freshly isolated, semipurified blood T- cells to PHA (TABLE 2.1C).

TABLE 2.1

T-CELL STIMULATORY ACTIVITY OF MoCM AND LyCM IN LIQUID CULTUR

2.1A)THYMOCYTES

Thymidine incorporation (c.p.m. x 10^3)

	expt.1	expt.2	expt.3
medium	0.4	0.3	1.5
PHA (0.5%)	1.0	0.3	2.7
MoCM (15%)	1.0	1.5	3.4
MoCM+PHA	5.7	6.1	31.7
LyCM (50%)	3.9	11.5	25.8
LyCM+PHA	4.2	n.t	n.t
LyCM+MoCM	n.t	87.3	85.7

2.1B)CULTURED T-CELLS (T-CFCs)

Thymidine incorporation (c.p.m. x 10^3)

	expt.1	expt.2	expt.3
medium	0.7	0.5	0.4
PHA (0.5%)	1.0	0.9	1.0
MoCM (15%)	1.4	0.5	0.7
MoCM+PHA	1.3	0.6	0.5
LyCM (50%)	28.7	28.1	36.4
LyCM+PHA	22.9	35.7	34.6
LyCM+MoCM	n.t.	32.7	41.3

continued on following page

TABLE 2.1 (continued)

T-CELL STIMULATORY ACTIVITY OF MoCM AND LyCM IN LIQUID CULTURE

2.1C) BLOOD MONONUCLEAR CELLS AND T-CELLS

Thymidine incorporation (c.p.m. x 10^3)

	expt.1		expt.2	
	MC	T	MC	T
medium	0.5	1.5	0.6	0.9
PHA(0.5%)	24.0	7.3	36.5	8.4
MoCM	0.4	2.1	0.8	1.0
MoCM+PHA	29.9	28.2	41.8	29.6
LyCM	24.0	18.5	31.7	29.8
LyCM+PHA	25.9	42.8	33.8	35.3

Cultures were carried out and thymidine incorporation was measured as described in materialsand methods. Figures are the mean values of duplicate or triplicate cultures in each experiment. n.t.= not tested

TABLE 2.2

ENHANCEMENT OF THYMOCYTE MITOGENESIS BY MOCM

	Thymidine incorporation (c.p.m.)
medium alone	403±160
PHA (1%)	609±280
PHA+MoCM 50%	1656±757
25%	5259±285
12%	8315±2264
6%	5618±1379
MoCM 12% (no PHA)	1321±279

Figures are the mean values ± 1S.D. of quadruplicate cultures, in a representative experiment. Similar results were obtained with thymocytes in other experiments.

In contrast to MoCM, LyCM not only enhanced thymocyte and blood T- cell proliferation but also stimulated proliferation of cultured T-cells (TABLE 2.1B). In the majority of these experiments target cells for testing for IL-2 activity were T-colony cells (T-CFCs) harvested from 6-7 day old primary cultures. These cells showed responses to PHA and MoCM for a short period after harvesting (48hrs), but by 96 hours these responses were either markedly reduced or abolished, whereas there remained a vigorous proliferative response to LyCM (TABLE 2.3). Long term proliferation of T-CFCs (2-4 weeks after harvesting) could be maintained by regular feeding, every 3-4 days, with fresh LyCM. In this respect no differences could be observed between cultured T-CFCs and cultured T-cells produced by PHA activation in liquid culture of blood MC with subsequent maintenance in LyCM (FIG 2.1). Nor could any differences be observed between conventionally cultured blood T-cells and T-CFCs either in their proliferative responses to PHA and CM (TABLE 2.4) or in their phenotype (TABLE 2.5), although conventionally cultured T-cells were found to retain greater resposiveness to PHA than T-CFCs (TABLE 2.6). The response of T-CFCs to LyCM was dependent on the concentration of CM and increased to a maximum at 25% concentration (with spleen LyCM). The response of cultured T- cells was not increased by the addition of PHA to cultures (TABLE 2.7).

TABLE 2.3

EFFECT OF INCUBATION TIME ON PROLIFERATION OF T-CFCs

	Thymidine incorporation (c.p.m.x10^3)		
Incubation time:	48hours	72hours	96hours
medium	39.6±10.0	0.2±0.1	0.4±0.1
PHA 1%	128.7±6.8	0.9±0.1	0.4±0.2
MoCM 15%	90.6±13.0	0.7±0.5	0.4±0.1
MoCM+PHA	122.0±11.7	4.5±0.3	0.8±0.4
LyCM 50%	166.3±9.3	89.7±10.6	63.0±6.4
LyCM+PHA	137.5±15.9	94.5±19.9	54.4±9.4

Figures are the mean values ± 1S.D of triplicate cultures. T colony cells (T-CFCs) were harvested from primary cultures after 6 days and then incubated for 48, 72 or 96 hours in suspension culture at 10^5cells/ml in medium containing RPMI 1640 with 10% human serum supplemented with PHA or conditioned media, as shown in the table. Cells were pulsed with 3H-thymidine for the last four hours of culture, and cells were then harvested and thymidine incorporation was measured as described in materials and methods.

TABLE 2.4

COMPARISON OF PROLIFERATIVE RESPONSES TO CONDITIONED MEDIA BY T-CFCs AND CONVENTIONALLY CULTURED T-CELLS

Thymidine incorporation (c.p.m)

	T-CFCs	Cultured T-cells
medium	1264±256	496±315
PHA 0.5%	2383±1459	1858±650
MoCM 15%	927±103	424±357
MoCM+PHA	2204±100	1835±532
LyCM 50%	22161±1250	12639±771

Figures are the mean value ± 1S.D of triplicate cultures. In this experiment blood MC from a single individual were cultured to provide colony cells - T-CFCs (from standard one step cultures) and conventionally activated T-cells by incubating blood MC in suspension with 1% PHA for three days. T-CFCs and conventionally activated T-cells were subsequently cultured in LyCM, with regular feeding every three to four days. (see FIG 2.1) Two weeks after the initiation of cultures proliferative responses to CM were assayed as described in materials and methods. Similar results have been obtained in two subsequent experiments.

TABLE 2.5

COMPARISON OF PHENOTYPE OF T-CFCs AND CONVENTIONALLY CULTURED T-CELLS

	% positive cells			
	E-rosettes	OKT3	OKT4	OKT8
T-CFCs	96.0	98.5	56.5	44.0
Cultured T-cells	92.0	93.0	53.0	43.0

T-CFCs (colony cells) and conventionally cultured T-cells were obtained from two week cultures as described in table 2.4.

TABLE 2.6

COMPARISON OF PROLIFERATIVE RESPONSES OF T-CFCs AND CONVENTIONALLY CULTURED T-CELLS

	Thymidine incorporation (c.p.m.)	
	T-CFCs	Cultured T-cells
medium	360±212	575±327
PHA 0.5%	1275±605	1260 +111
LyCM 50%	16175±5329	27025±1707

Figures are the mean values ± 1S.D. of 12 experiments using freshly isolated T-CFCs and 4 experiments using conventionally activated T-cells that had been maintained in culture with LyCM for two to three weeks.

TABLE 2.7 RESPONSE OF T-CFCs TO LYCM

Concentration of LyCM(%)	Thymidine incorporation (c.p.m.) medium	+PHA
0	1071±313	1402±480
6	3693±1210	4458±1531
12	12756±1285	14291±994
25	23972±3766	24594±1973
50	25367±2513	25212±1057

Figures are the mean values ± 1S.D of triplicate cultures.

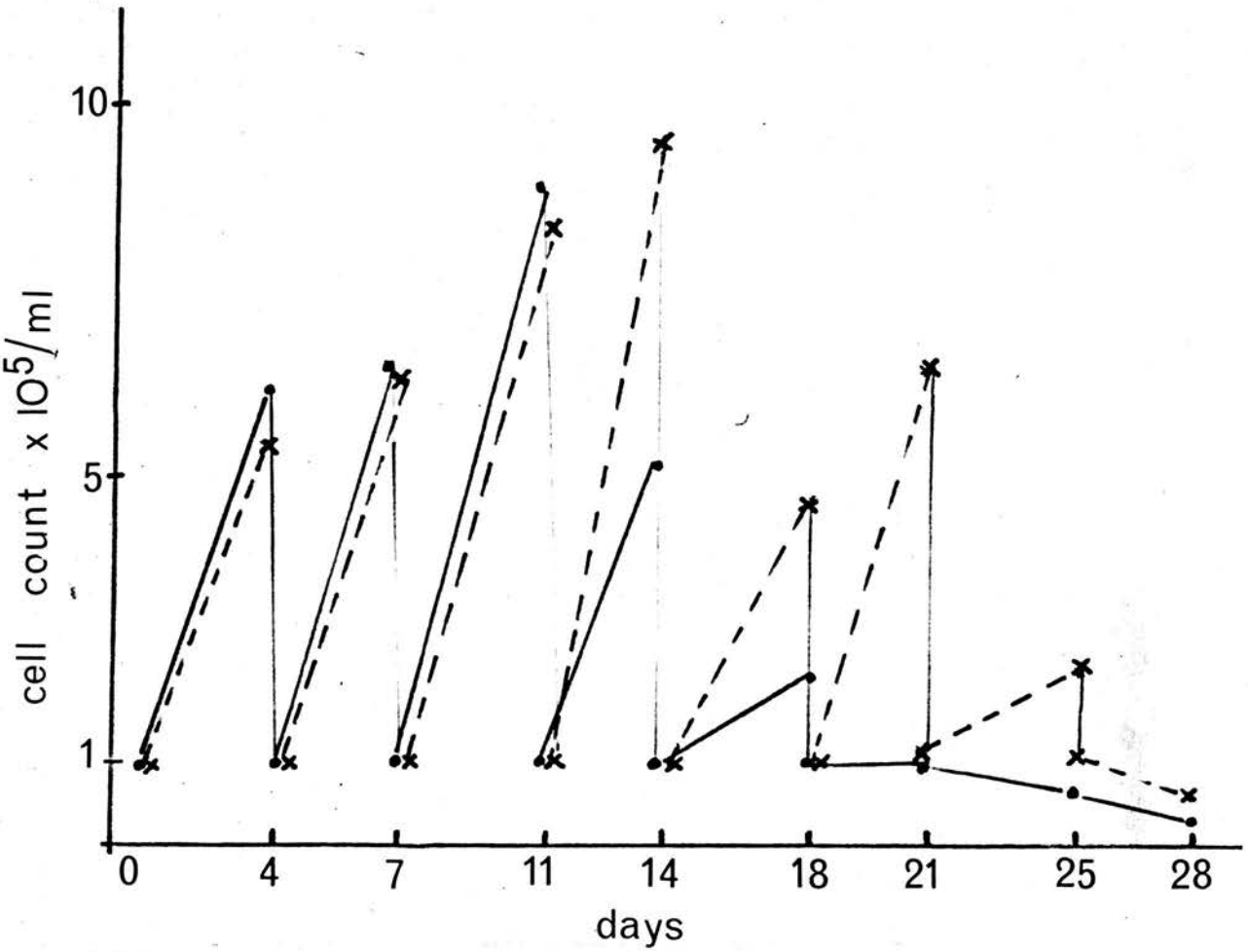

FIGURE 2.1 LONG TERM CULTURE OF T-CFCs AND CONVENTIONALLY ACTIVATED T-CELLS

T colony forming cells (T-CFCs ×- - - -×) and T cells activated by conventional culture in suspension with PHA (•———•) were prepared as described in table 2.4. Cells were initially suspended at 1 x 10^5/ml in RPMI 1640 containing 10% human serum and 50% LyCM (growth medium). Cells were cultured in 5ml volumes in 25ml Falcon tissue culture flasks. Every three to four days cells were counted, and resuspended in fresh growth medium at 1 x 10^5cells/ml.

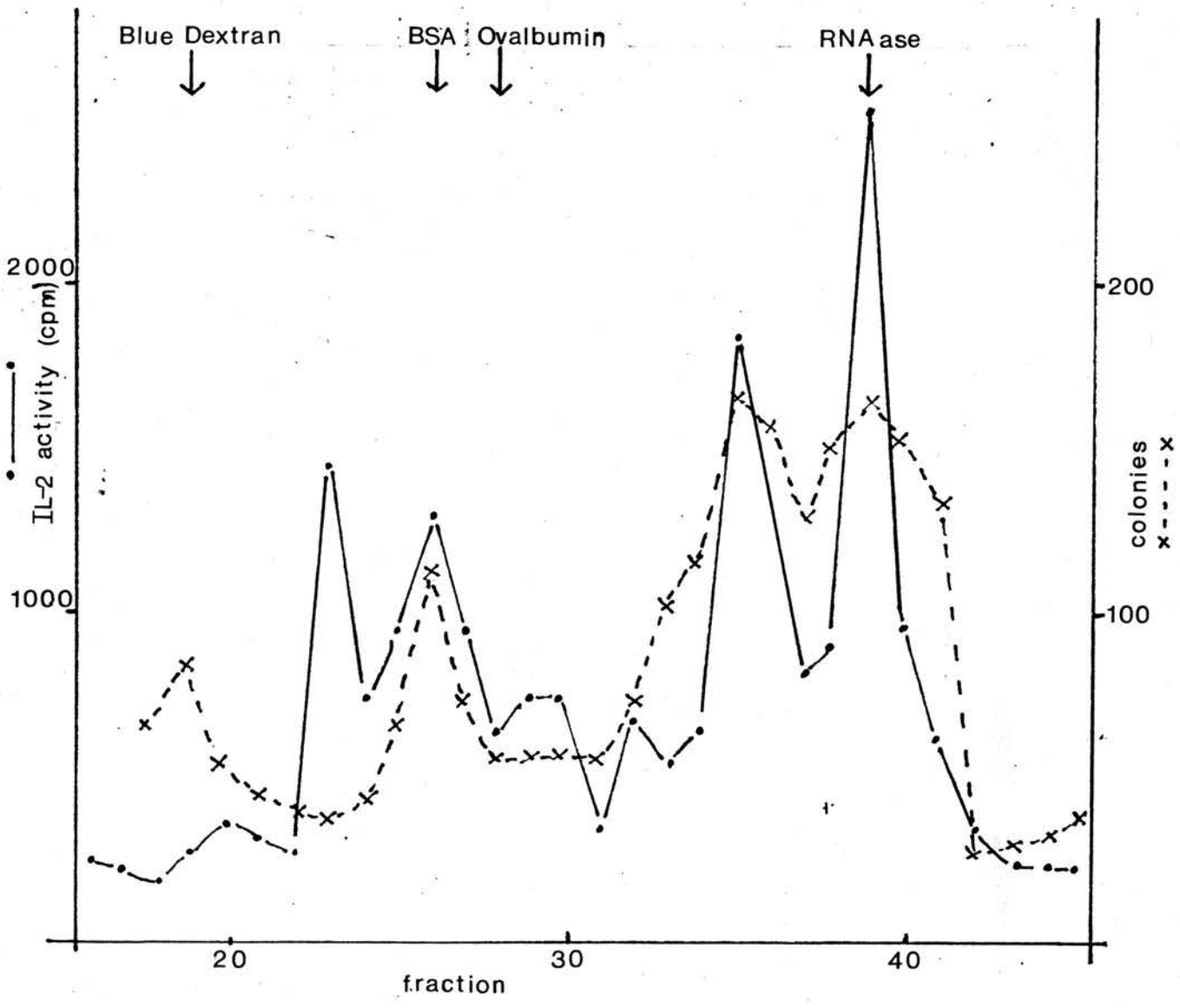

FIGURE 2.2 SEPHADEX G-100 FILTRATION OF LYCM
Points represent the mean response of triplicate cultures.
IL-2 activity was assessed as described in methods by culturing T-CFCs with test medium at 50% v/v concentration.
Colony stimulating activity was measured by culturing blood MC at 1 x 10^5 cells/well in standard one step primary colony forming assays, with test medium at 50% v/v concentration and PHA 1% v/v in the underlayer. No colony growth was obtained in the absence of PHA.

Partial purification of LyCM by Sephadex G-100 gel filtration showed several peaks of activity. The maximum IL-2 activity was in fractions, corresponding to the reported mol.wt of interleukin 2 of 15,000 daltons (FIG 2.2). Partially purified IL-2 stimulated cultured T- cells, but was not mitogenic for freshly isolated blood T-cells in the absence of PHA (TABLE 2.8).

TABLE 2.8

STIMULATION OF T-CELLS BY PARTIALLY PURIFIED IL-2 (ppIL-2)

	Thymidine incorporation (c.p.m.x10^3)	
	T-cells	T-CFCs
medium	0.8	0.6
PHA	10.3	1.2
LyCM	39.4	22.0
ppIL-2	1.0	8.6
ppIL-2+PHA	21.3	9.8

Figures are the mean values of duplicate cultures. T-cells were isolated from blood MC by rosetting with sheep erythrocytes, as described in materials and methods. T-cells consisted of 96% E-rosette positive cells. T-cells were cultured at 1x10^6 /ml for three days and T-CFCs at 1x10^5 cells/ml for four days.

With medium renewal every three to four days both T- CFCs and conventionally cultured blood T- cells could usually be maintained for 3-4 weeks in rapid proliferation (with a doubling time of about 24- 36 hours). After this most cultures lost responsiveness to LyCM and could not be induced to proliferate further (FIG 2.1). The maximum period over which T-CFCs were maintained was 12 weeks in continuous culture before loss of responsiveness to LyCM. Withdrawal of LyCM from cultured T- CFCs resulted in rapid cell death, as evidenced by a fall in cell count and morphological changes such as chromatin condensation and nuclear and cellular fragmentation. These changes appeared within 24 hours of LyCM withdrawal. In these cultures cell viability assessed by trypan blue exclusion was not a reliable indicator of cell death and remained high (>90%) for several days after withdrawal of LyCM, even though suspensions stained with acridine orange or by the Feulgen method showed nuclear pyknosis and fragmentation (TABLE 2.9.).

TABLE 2.9

THE EFFECT OF WITHDRAWAL OF LyCM FROM CULTURED T-CFCs

cell count ($x10^5$/ml)		%pyknotic cells		%viability (trypan blue)	
+LyCM(50%)	-LyCM	+LyCM	-LycM	+LyCM	-LyCM
Day of culture:					
0 5.0	5.0	23	23	96	96
1 7.8	6.6	15	27	100	100
2 16.5	6.3	11	34	100	99
3 25.2	6.1	27	59	100	99
Cultures adjusted to 5x10^5/ml and resuspended in fresh medium					
4 4.1	2.8	22	60.5	100	100
5 6.5	1.5	23	86.0	100	97
6 9.0	1.6	14.5	63.0	100	100
7 11.2	0.1	7	not counted	100	not counted

Figures are the mean values of duplicate cultures. Freshly harvested T- CFCs were cultured in 17mm diameter multiwell plates under the conditions described in the table.

LyCM, prepared by PHA stimulation of blood, tonsil and lymph node lymphocytes were also found to be effective in stimulating proliferation of cultured T-cells. The highest activities were found in supernatants from unfractionated MC; CM from T- enriched cells (prepared by E-rosetting) showed slightly lower levels of activity than MC. The activity of these CM was increased when they were prepared from cells to which MoCM had been added. This enhancement was more marked with T-enriched cells than unfractionated MC (TABLE 2.10A). An unexpected finding was that T-depleted cell suspensions (<5% E+) produced active supernatants, although the activity of these was less than that found in CM from MC or T-enriched cells. PHA stimulated thymocyte culture supernatants showed only low levels of IL-2 activity. This was increased by culturing thymocytes with MoCM although activity of thymocyte CM was still much lower than that present in blood MC or spleen MC culture supernatants (TABLE 2.10B). Supernatants from cultured T-cells, stimulated with PHA and/or MoCM did not contain any IL-2 activity (TABLE 2.10C).

TABLE 2.10 PRODUCTION OF IL-2 BY MONONUCLEAR CELLS

2.10A) IL-2 PRODUCTION BY BLOOD, TONSIL AND LYMPH NODE

	IL-2 activity Thymidine incorporation (c.p.m.x10^3)		
CM prepared from	blood	lymph node	tonsil
unfractionated MC + PHA	35.0	84.3	92.7
+PHA + MoCM	42.1	115.1	103.2
T-enriched (E+) +PHA	30.2	41.1	67.2
+PHA + MoCM	62.2	59.0	93.6
T-depleted (E-) +PHA	n.t	12.6	57.3
+PHA + MoCM	n.t	35.0	71.0
controls: medium	0.7	0.5	2.2
PHA (0.5%)	1.0	0.5	4.1
PHA + MoCM (15%)	1.4	1.4	1.5
spleen LyCM (50%)	89.9	135.0	102.3

Figures are the mean values of triplicate cultures. MC were fractionated into T-enriched and depleted populations by rosetting with sheep red blood cells. All T-enriched cells contained >95% E+ cells and <1% NSE positive cells; T-depleted suspensions contained <5% E+ cells and between 10-15% NSE positive cells. (table 10 continued on next page)

2.10B) IL-2 PRODUCTION BY THYMOCYTES

	IL-2 activity Thymidine incorporation (c.p.m.x10^3)		
Conditioned medium from;thymus	1	2	3
thymocytes + PHA	1.6	1.6	1.6
+PHA + MoCM	4.5	7.4	3.6
controls: medium	0.5	0.5	0.5
PHA 0.5%	0.6	0.9	0.9
PHA + MoCM 15%	1.0	0.6	0.6
spleen LyCM 50%	39.9	28.2	28.2

C) IL-2 PRODUCTION BY T-COLONY FORMING CELLS

	IL2 activity Thymidine incorporation (c.p.m.x10^3)			
Conditioned medium from; T-CFCs	1	2	3	4
T-CFCs + PHA	0.4	0.4	0.8	0.7
+PHA + MoCM	n.t.	n.t.	0.9	0.6
controls: medium	0.3	0.3	0.8	0.8
PHA 0.5%	0.7	0.7	1.2	1.0
PHA + MoCM 15%	n.t.	n.t.	1.2	0.7
LyCM 50%	12.8	12.8	16.4	11.7

These studies demonstrated that, by conventional bioassay, MoCM exhibited IL-1 activity and LyCM exhibited IL-2 activity: MoCM showed a direct mitogenic effect on thymocytes, enhanced PHA induced thymocyte and T-cell mitogenesis and enhanced IL-2 production by thymocytes and blood T-cells. MoCM did not possess demonstrable IL-2 activity : proliferation of cultured T- cells was not supported. LyCM in contrast was able to maintain relatively long term proliferation of cultured T-cells that no longer respond to MoCM and PHA and therefore showed IL-2 activity.

1B. ENHANCEMENT OF T-COLONY FORMATION BY CONDITIONED MEDIA

Colony formation by blood MC

Addition of either MoCM or LyCM to culture underlayers enhanced colony formation by blood MC, in a dose dependent manner: optimum enhancement with MoCM was obtained with 12% MoCM and with 25% spleen LyCM (TABLE 2.11).

TABLE 2.11

ENHANCEMENT OF COLONY FORMATION BY MOCM AND LYCM

Concentration (%) of CM in underlayer	Colonies	
	MoCM	LyCM
0	17±12	
3	24±27	35±33
6	78±22	85±14
12	112±13	156±26
25	79±12	185±53
50	64±20	151±32

Cells were cultured at 1 x 10^5 /well. Figures are the mean value + 1S.D of triplicate cultures.

Enhancement of colony formation was dependent on the presence of PHA. With MoCM no colony formation occurred in the absence of PHA. With LyCM some colony formation was observed in cultures to which PHA had not been added, but many more were formed in cultures containing both LyCM and PHA (TABLE 2.12). Unlike MoCM, LyCM contains some PHA, albeit at concentrations suboptimal for colony production. This probably accounts for colony growth in medium supplemented with LyCM alone. In an experiment using partially purified IL- 2, prepared by gel filtration, colony formation only occurred in the presence of PHA (FIG 2.2).

The enhancing effect of both CM was most marked at low cell concentrations ($0.5\text{-}1.0 \times 10^5$ cells/well). At higher cell concentrations more variable effects were seen: some donors' MC showed no increase or a small reduction in colony numbers (FIG 2.3).

As well as increasing the number of colonies MoCM and LyCM were also observed to increase colony size. This effect on colony size was most marked at low cell concentrations when LyCM was present in the cultures. With MoCM the effect on colony size was most marked at high cell concentrations (FIG 2.4).

TABLE 2.12

ENHANCEMENT OF BLOOD MC COLONY FORMATION BY CONDITIONED MEDIA

	Colonies		
	exptl	expt2	expt3
underlayer			
PHA (0.5%)	79±9	195±6	173±69
PHA + MoCM (15%)	128+6	312±29	342±15
PHA + LyCM (25%)	108±16	247±14	232±12
PHA + MoCM + LyCM	169±4	305±29	348±4
no PHA			
MoCM (15%)	0	0	0
LyCM (25%)	44±14	165±25	137±16
MoCM+LyCM	67±11	188±40	173±23

Cells were cultured at 1.5 x 10^5/well. Figures are the mean value ± 1S.D of triplicate cultures. These three experiments are representative of a larger series.

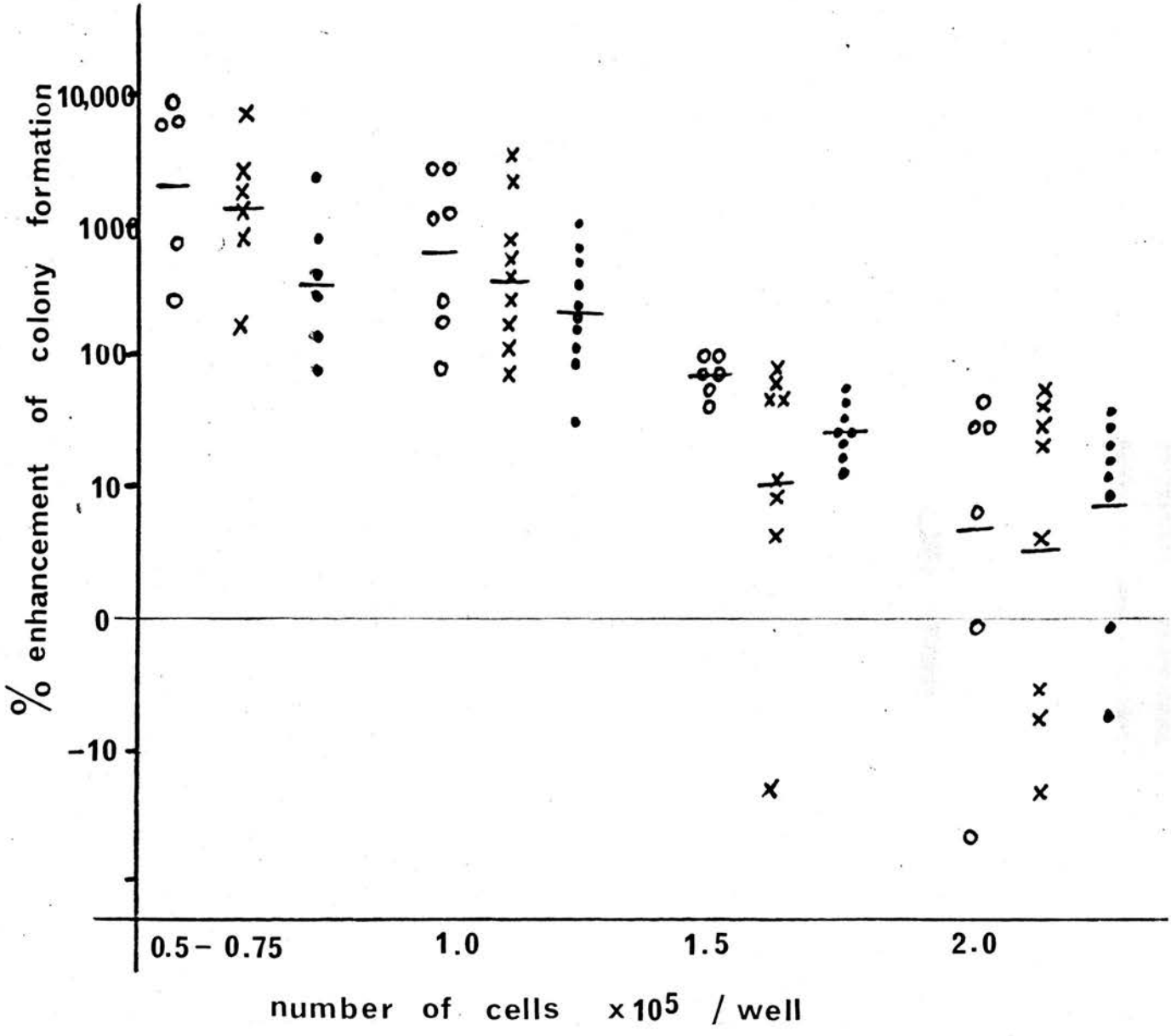

FIGURE 2.3 ENHANCEMENT OF COLONY FORMATION BY MOCM AND LYCM AT DIFFERENT CELL CONCENTRATIONS

Each point represents the % enhancement of colony formation seen when blood MC were cultured on underlayers containing either MoCM (×), LyCM (•) or both MoCM and LyCM (o).

$$\%\text{enhancement} = \frac{\text{no. colonies with CM} - \text{no. colonies with PHA}}{\text{no. colonies with PHA}} \times 100$$

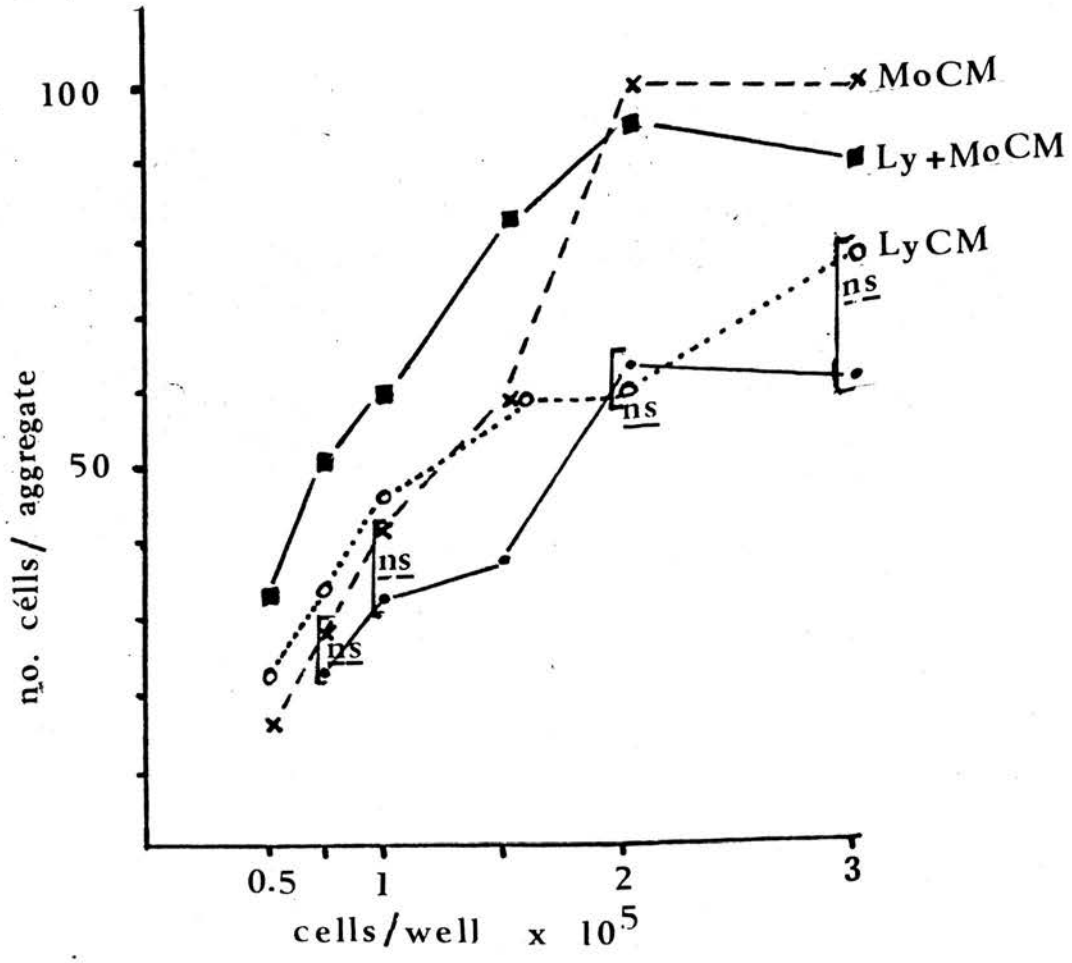

FIGURE 2.4 THE EFFECT OF MOCM AND LYCM ON COLONY SIZE
Each point represents the mean size of 50-100 cell aggregates (colonies and clusters) developing in cultures either with PHA alone (•——•), or in cultures supplemented with MoCM (15%v/v) (×- - -×) orLyCM (25%v/v) (o·····o) or both MoCM and LyCM (■——■). Aggregate size on CM underlayers was significantly greater than with PHA alone (P<.01 Mann WHitney U test), except where shown (n.s.=not significant).

Colony formation by blood T-cells purified by E-rosetting

Mononuclear cells were fractionated into T-enriched and T-depleted populations by rosetting with sheep red blood cells (E-rosetting) (TABLE 2.13).

T-enriched cell populations (>96%E+) showed poor or no colony formation on underlayers with PHA alone. However when MoCM, or LyCM was included in culture underlayers colony formation was comparable to that of unfractionated MC (TABLE 2.14). Further experiments showed that colony formation by enriched T- cells could also be induced by culturing cells on underlayers containing T- depleted blood MC (i.e. B-cells and monocytes) or on adherent cell underlayers (i.e. monocytes) (TABLES 2.14 and 2.15).

T-depleted cell suspensions (<5%E+) produced either no colonies (2/4 experiments) or only a small number of colonies, regardless of supplementation of the medium with MoCM and LyCM.

TABLE 2.13

FRACTIONATION OF BLOOD MC BY ROSETTING WITH SHEEP RED BLOOD CELL

	% positive cells		
	E+	N.S.E.+	SIg+
Experiment 1:MC	47	10	n.d.
T-enriched	97	0.5	n.d.
T-depleted	2	10	n.d.
Experiment 2:MC	68	13	n.d.
T-enriched	96	1	n.d.
T-depleted	1.5	23	n.d.
Experiment 3:MC	55	10	15
T-enriched	98	0.5	0.5
T-depleted	3	22	45
Experiment 4:MC	52	12	n.d.
T-enriched	93	1	n.d.
T-depleted	5	19	n.d.

n.d. = not done

TABLE 2.14 COLONY FORMATION BY T-CELL ENRICHED AND DEPLETED BLOOD MC POPULATIONS ON CM UNDERLAYERS

	number of colonies		
Underlayer:	PHA	+LyCM	+MoCM
Experiment 1			
MC	142±8	184±10	153±9
T-enriched	23±4	126±6	103±7
T-depleted	0	0	0
Experiment 2			
MC	183±26	197±16	203±28
T-enriched	18±5	234±35	188±17
T-depleted	6±3	21±12	132±2
Experiment 3			
MC	112±12	143±17	131±9
T-enriched	0	287±9	165±9
T-depleted	0	0	0

In experiment 1 cells were cultured at 2 x 10^5 cells/well on standard underlayers (no RBC). In experiments 2 and 3 cells were cultured on RBC containing underlayers at 1 x 10^5 cells/well.

TABLE 2.15

COLONY FORMATION BY T-CELL ENRICHED AND DEPLETED BLOOD MC POPULATIONS ON ADHERENT CELL UNDERLAYERS

	number of colonies		
Underlayer:	PHA	MoCM	Adherent cells
Experiment 4			
$2x10^5$ cells/well			
MC	178±55	197±11	189±35
T-enriched	40±4	335±12	305±34
T-depleted	15±3	27±8	17±4
$1x10^5$ cells/well			
MC	62±22	63±5	63±12
T-enriched	17±7	111±4	100±11
T-depleted	0	0	0

Figures are the mean values ± 1 S.D. of triplicate cultures. Cells were cultured on standard underlayers (no RBC) supplemented either with MoCM (15%v/v) or with adherent cells. Underlayers were prepared as described in materials and methods.

Colony formation by blood MC treated with monoclonal antibodies

Blood MC were treated with the anti T-cell antibodies OKT3,OKT4 and OKT8 and complement in order to deplete MC suspensions of the respective T- cell subset (TABLE 2.16).

TABLE 2.16

DEPLETION OF T-CELL SUBSETS FROM BLOOD MC BY TREATMENT WITH MONOCLONAL ANTIBODIES AND COMPLEMENT

	% positive cells (expt2)		
	OKT3	OKT4	OKT8
untreated	67	43	21
OKT3+complement	15	11	4
OKT4+complement	35	4	28
OKT8+complement	48	39	3

When these subset depleted cell suspensions were cultured in agar, colony formation was greatly reduced in OKT3, OKT4 and OKT8 depleted suspensions. Subset depleted cultures supplemented with LyCM showed increased numbers of colonies, although still less than control cultures. Cultures treated with complement alone grew normally. Treatment with the anti-thymocyte antibody OKT6 had no effect on colony formation in respose to PHA but did reduce the number of colonies produced on underlayers supplemented with LyCM (TABLE 2.17).

TABLE 2.17

INHIBITION OF COLONY FORMATION BY TREATMENT OF BLOOD MC WITH MONOCLONAL ANTIBODIES AND COMPLEMENT

	Colonies			
	expt.1		expt.2	
Underlayer:	PHA	LyCM	PHA	LyCM
OKT3+complement	29	128	36	146
OKT4+complement	107	119	37	141
OKT8+complement	145	134	80	177
OKT6+complement	215	219	185	200
untreated MC	218	281	175	285
+complement	226	304	175	283

Figures are the mean values of duplicate cultures in two experiments using MC from two different donors.

Treatment of blood MC with the anti-monocyte antibody OKM1 reduced colony formation by around 50%. This inhibition was completely reversed by the addition of MoCM to cultures (TABLE 2.18).

TABLE 2.18

INHIBITION OF COLONY FORMATION BY TREATMENT OF BLOOD MC WITH OKM-1 AND COMPLEMENT

	colonies					
	expt 1.		expt 2.		expt 3.	
underlayer	PHA	MoCM	PHA	MoCM	PHA	MoCM
untreated MC	218	261	175	248	130	218
+complement	226	243	175	263	122	220
OKM-1+complement	107	224	45	271	74	208

Figures are the mean values of duplicate cultures.

In an attempt to determine whether the reduction in colony formation, following treatment of MC with anti T-cell antibodies, was solely due to removal of colony forming precursors or secondary to a loss or reduction of growth factor production by accessory cells, the release of IL-2 activity by OKT3, OKT4 and OKT8 treated cells was measured. These experiments showed that depletion of OKT3+ and to a lesser extent of OKT4+ cells reduced IL-2 production but depletion of OKT8+ cells moderately increased it (TABLE 2.19).

TABLE 2.19

INHIBITION OF IL-2 PRODUCTION BY BLOOD MC TREATED WITH MONOCLONAL ANTIBODIES AND COMPLEMENT

	IL-2 activity (c.p.m.)	
	expt 1.	expt. 2
Culture supernatant from:		
untreated MC	5672±801	8932±1280
OKT3+complement	216±81	1572±82
OKT4+complement	1238±223	5887±145
OKT8+complement	6184±224	13478±922
medium alone	743±224	
medium+PHA	983±741	

Culture supernatants were produced by culturing cells at 1 x 10^6/ml in RPMI 1640, containing 1% PHA and 1% serum, for 48 hours. Supernatants from these cultures were assayed for IL-2 activity by culturing with T-CFCs as described in materials and methods. Supernatants were tested at 50% v/v concentration. Figures are the mean value ± 1S.D. of triplicate cultures.

Colony formation by thymocytes

Thymocytes showed little or no colony formation with PHA alone but could be induced to form colonies when cultured on underlayers containing either MoCM or LyCM. With MoCM colony formation was poor except at high cell concentrations (TABLE 2.21). Thymocyte colonies and colony cells were morphologically identical to blood MC derived colonies and consisted of mature OKT3+ T cells, and not immature, OKT6+ thymic precursors. (TABLE 2.20).

TABLE 2.20

PHENOTYPE OF THYMIC COLONY CELLS

	%positive cells		
	E+	OKT3+	OKT6+
Thymocytes	89	21	86
Thymic colony cells	94	98	1.0

TABLE 2.21 COLONY FORMATION BY THYMOCYTES

		number of colonies			
Underlayer:		PHA	LyCM	MoCM	LyCM+MoCM
Experiment 1 (freshly isolated thymocytes)					
cells/well	4	4±3	207±12	22±16	228±8
$x10^5$	2	1±1	112±6	5±2	105±27
	1	0	46±7	0	19±11
	0.5	0	1±1	0	0
Experiment 2 (cryopreserved thymocytes)					
cells/well	4	41±18	154±14	129±13	229±20 (23±5)
$x10^5$	2	13±4	95±10	38±10	141±15 (5±2)
	1	0	21±10	2±2	100±21 (0)
	0.5	0	0	0	34±7 (0)
Experiment 3 (cryopreserved thymocytes)					
cells/well	4	0	159±15	18±5	177±6
$x10^5$	2	0	55±14	3±1	63±27
	1	0	27±4	0	29±4
	0.5	0	12±7	0	18±11

In experiment 1 cells were cultured on standard underlayers (no RBC). In experiments 2 and 3 cells were cultured on RBC containing underlayers. In experiments 2 and 3 no colonies were obtained on standard underlayers except in cultures supplemented with both LyCM and MoCM (figures in parentheses for experiment 2).

Colony formation by T-colony forming cells in secondary culture

T-colony forming cells (T-CFCs) harvested from primary culture and replated as a single cell suspension in agar showed no growth in the absence of PHA or conditioned media. With PHA alone or with MoCM some of the seeded cells proliferated within the first 1-2 days to form small clusters, up to 10 cells in size. In most cultures these failed to enlarge further and after the second day of culture, cells in the clusters began to degenerate. In contrast cultures supplemented with LyCM showed rapid proliferation of cells with colonies (>40 cells) developing within 4-5 days of the initiation of cultures (TABLE 2.22).

Colony formation in response to LyCM, in secondary cultures, was dose dependent, but unlike primary cultures was not increased by adding PHA to cultures (TABLE 2.23).

TABLE 2.22

A) COLONY FORMATION IN SECONDARY CULTURE

	colonies				
	expt.1	2	3	4	5
medium alone	0	0	11±15	0	0
+PHA	0	0	147±13	7±6	0
MoCM	0	0	0	0	0
+PHA	0	0	5±6	12±4	0
LyCM	292±19	56±15	71±23	138±11	93±11
+PHA	396±18	130±57	284±6	108±12	190±17
LyCM+MoCM	-	not tested	-	127±31	97±15
+PHA				139±27	184±21

B) COLONY FORMATION IN SECONDARY CULTURE ON RBC UNDERLAYERS

	colonies					
	6		7		8	
expt:	-	+RBC	-	+RBC	-	+RBC
medium alone	0	0	0	0	0	0
+PHA	0	30±8	21±7	35±7	0	15±4
LyCM	93±6	129±17	130±17	111±19	163±24	121±7
+PHA	91±11	110±9	138±23	145±12	159±26	134±9

All cultures at 1 x 10^5cells /well. Figures are the mean values ± 1 S.D. of triplicate cultures.

TABLE 2.23

RESPONSE OF T-CFCs TO LYCM IN SECONDARY CULTURE

Concentration (%) of LyCM in the underlayer	Colonies	
	-PHA	+PHA (1%)
0	0	0
6	23±11	18±9
12	61±16	72±8
25	132±25	158±18
50	141±15	151±19

Figures are the mean colony count ± 1 S.D. of triplicate cultures.

2. KINETIC ANALYSIS OF COLONY FORMATION

The requirement for cell interactions in colony formation by blood MC in primary culture and T-CFCs in secondary culture was investigated by mathematical analysis of the relationship between colony numbers and plating cell concentration, as detailed in materials and methods. Briefly, the number of interacting cells was determined by calculating the slope (b) of regression lines obtained from a plot of log.colonies/log.cells per well. By performing experiments in which CM were added to cultures and comparing regression coefficients (b) on CM underlayers with standard underlayers (PHA alone) it is possible to determine whether CM reduce the requirement for cell interactions (i.e. reduce b) and substitute for accessory cells.

Kinetic analysis of blood MC colony formation in primary culture

The results of a typical experiment are shown in TABLE 2.24. On standard underlayers (PHA alone) the slope of the regression line (log.colonies/log.cells per well) was 3.1, suggesting a requirement for three interacting cells. With underlayers containing both MoCM and LyCM the slope of the regression line was 1.0, i.e. the fall in plating efficiency with falling cell concentration seen in cultures with PHA alone was abolished and a simple arithmetic relationship between the number of colonies and the number of cells plated

existed, suggesting that under these conditions colony formation was apparently independent of cell interactions. With underlayers containing either MoCM or LyCM alone slopes of regression lines were 1.7 for MoCM and 2.3 for LyCM indicating a partial reduction in the requirement for interactions from 3, on standard underlayers, to around 2 in the presence of one CM. No colonies formed with MoCM in the absence of PHA, but with LyCM (containing residual PHA) and LyCM+MoCM b-values were similar in cultures without added PHA to those with PHA although the plating efficiency was reduced (TABLE 2.24).

In a series of experiments using blood MC from different donors similar results were obtained to those described above. Significant differences in b values were found between cultures on underlayers with PHA alone and underlayers supplemented with CM. (TABLE 2.25 and FIG 2.5).

The above experiments were all performed on underlayers without RBCs. Experiments in which kinetic analysis was applied to colony formation on RBC underlayers showed that inclusion of RBCs reduced the slopes of regression lines from 3 (PHA, no RBC) to 2 (PHA+1%RBC) (TABLE 2.26). Inclusion of MoCM or LyCM increased colony formation at lower cell concentrations and reduced the slope of regression lines to 1.5. However both CM were required, even in the presence of RBC to obtain a regression line with a slope of 1 (TABLE 2.26).

TABLE 2.24

KINETIC ANALYSIS OF COLONY FORMATION BY BLOOD MC IN PRIMARY CULTURE

	Number of colonies					
Underlayer: PHA	PHA	PHA	PHA		LyCM	
	+LyCM	+MoCM	+LyCM+MoCM	LyCM	+MoCM	
cells/well x 10^5						
3	562±75	541±20	485±70	439±48	223±10	255±8
2	380±84	474±18	399±42	383±48	176±42	194±22
1.5	173±69	232±12	342±15	348±4	137±16	173±23
1.0	126±21	169±33	248±18	228±66	148±5	126±12
0.75	45±9	82±18	126±29	170±58	70±22	115±6
0.5	1±1.5	6±2	17±15	65±29	4±6	48±6
b	3.1	2.3	1.7	1.0	1.9	0.8
r	0.89	0.90	0.88	0.93	0.81	0.95

The above table shows results from a typical experiment. Figures are the mean number of colonies ± 1 S.D. for triplicate cultures. b=slope of the regression line where logn = blogN + a; n=number of colonies, N=number of cells plated. r=correlation coefficient.

TABLE 2.25

KINETIC ANALYSIS OF COLONY FORMATION BY BLOOD MC IN PRIMARY CULTURE

(Experiments 1-9)

	b (regression coefficient)			
Underlayer	PHA	PHA+LyCM	PHA+MoCM	PHA+LyCM+MoCM
experiment:				
1	3.8	2.5	n.d	n.d
2	3.2	1.5	0.6	n.d
3	4.1	3.1	1.8	0.7
4	3.1	2.3	1.7	1.0
5	2.8	0.8	1.0	n.d
6	2.6	1.9	1.7	1.1
7	3.4	1.0	1.1	n.d
8	3.5	2.4	1.8	0.8
9	3.8	3.0	1.5	0.7
mean	3.4	2.1	1.4	0.9
Std.Error	0.2	0.3	0.1	0.1

Significant differences for b values were shown between PHA and LyCM ($p<.005$) and PHA and MoCM ($p<.005$) and also between LyCM or MoCM alone and LyCM+MoCM combined ($p<.005$). The difference between LyCM and MoCM was not significant ($0.1>p>0.05$). In the above experiments correlation coefficients (r) varied from 0.85- 0.99. n.d= not done

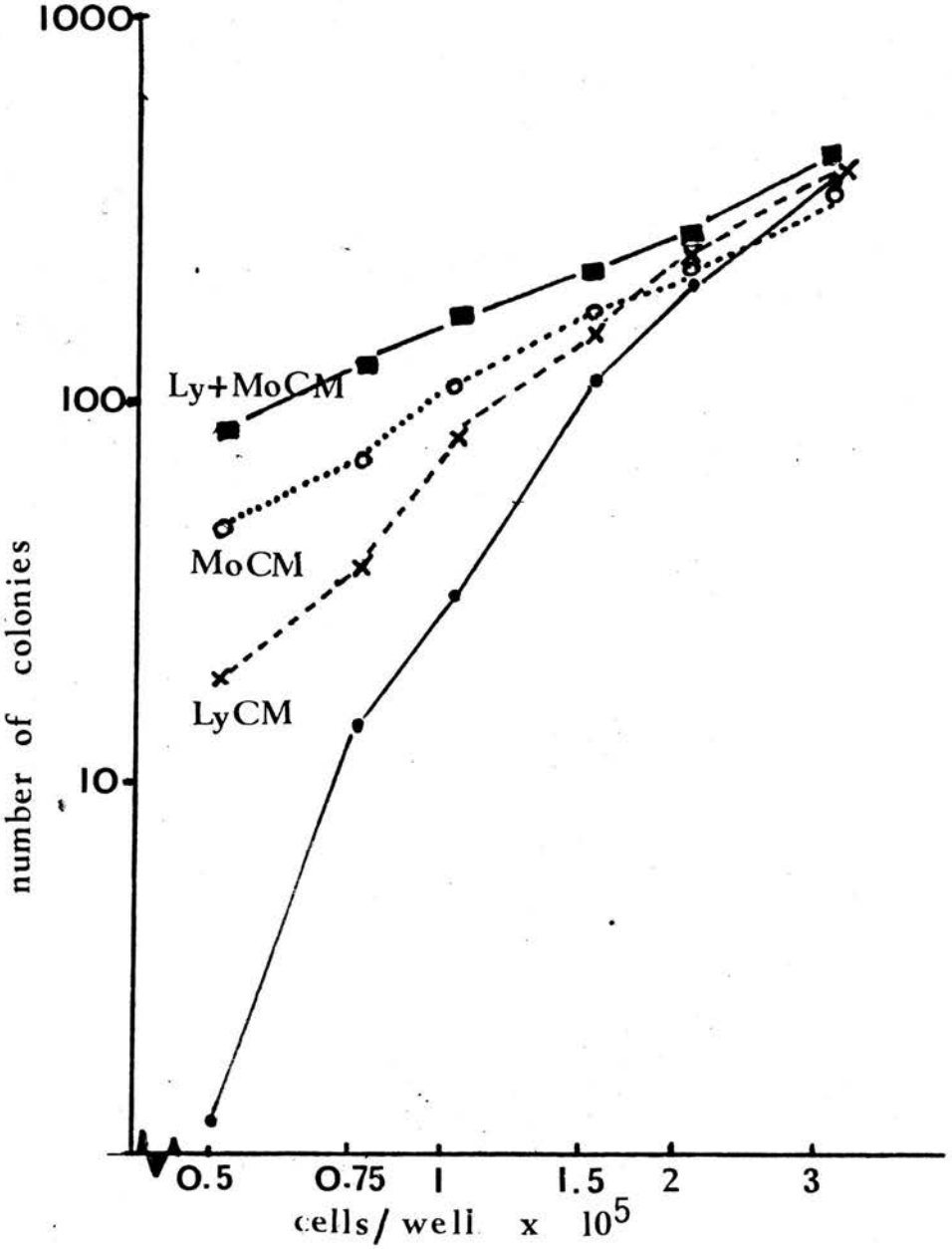

FIGURE 2.5 KINETIC ANALYSIS OF COLONY FORMATION
Each point represents the mean value of five to nine cultures using blood MC from different donors. Calculated slopes of regression lines gave values of 3.4 with PHA alone (•——•), 2.1 with LyCM (×- - -×), 1.4 with MoCM (o.....o) and 0.9 with both MoCM and LyCM (■——■).

TABLE 2.26 KINETIC ANALYSIS OF COLONY FORMATION BY BLOOD MC IN PRIMARY CULTURE WITH RBC CONTAINING UNDERLAYERS

	number of colonies WITHOUT RBC			
	PHA	LyCM	MoCM	LyCM+MoCM
cells/well x 10^5				
2.0	194±13	296±10	305±6	311±14
1.5	147±3	240±49	165±32	280±37
1.0	67±22	190±12	122±3	260±7
0.75	14±15	38±10	126±31	114±20
0.5	1±1	10±19	20±2	67±4
0.25		not done		
b	2.6	1.9	1.7	1.1
r	0.94	0.93	0.90	0.92

	number of colonies WITH RBC			
	PHA	LyCM	MoCM	LyCM+MoCM
cells/well x 10^5				
2	334±15	338±35	353±29	335±38
1.5	252±11	240±24	317±92	258±24
1.0	169±30	230±58	238±61	231±12
0.75	80±20	92±17	79±15	121±30
0.5	62±17	45±21	36±6	46±21
0.25	3±4	13±15	26±17	27±4
b	2.1	1.4	1.2	1.1
r	0.94	0.85	0.82	0.82

Figures are the mean values of triplicate cultures ± 1 S.D. This experiment was repeated using blood from a different donor and similar results were obtained.

Kinetic anlysis of secondary colony formation

When T-CFCs from primary cultures were recultured in agar, growth only occured in cultures with LyCM. Kinetic analysis of secondary colony formation with LyCM gave regression coefficients close to 1 i.e. the number of colonies forming was directly proportional to the number of cells plated and was independent of accesory cells in the presence of LyCM. The addition of PHA or RBCs to cultures did not significantly alter b-values, although cultures with RBCs showed increased plating efficiency (TABLE 2.27).

TABLE 2.27 KINETIC ANALYSIS OF SECONDARY COLONY FORMATION

cells/well x 10^5	Number of colonies 1.0	0.5	0.25	0.125	0.06	0.03	b	r
LyCM underlayer								
expt. 1	93±6	56±8	31±5	19±6	9±3	3±2	0.96	0.99
2	163±24	142±9	75±16	20±3	9±1	0	1.39	0.99
3	130±23	55±12	20±6	13±4	1±1	0	1.15	0.99
						mean=	1.16±0.22	
LyCM+PHA								
expt. 1	91±11	48±9	30±4	20±3	10±2	0	0.77	0.99
2	159±26	130±17	68±9	24±8	11±2	2±1	1.25	0.97
3	138±17	76±12	28±4	18±4	2±1	0	1.43	0.86
						mean=	1.15±0.34	
LyCM + RBC underlayer								
expt. 1	129±17	119±12	78±8	52±4	19±4	7±3	1.02	0.98
2	121±7	141±5	69±4	28±3	11±4	7±5	1.15	0.99
3	111±21	73±15	32±6	18±7	16±11	3±2	0.96	0.97
						mean=	1.04±0.09	
LyCM+PHA+RBC underlayer								
expt. 1	110±9	100±6	58±7	32±9	22±5	12±2	0.75	0.99
2	134±9	107±6	58±9	24±9	13±3	6±2	1.05	0.99
3	145±21	80±9	56±12	28±9	20±3	9±3	0.78	0.99
						mean=	0.86±0.16	

Figures are the mean values ± 1 s.d of triplicate cultures in three experiments, using T-CFCs from three different donors.

DISCUSSION

Demonstration of interleukin activity in CM

In the experiments described in this chapter two types of CM were prepared and assayed for T-cell stimulatory activity: MoCM was prepared from acute monocytic leukaemia cells, using a method described by Lachman et al (1978) for the preparation of CM with lymphocyte activating factor (IL-1) activity. LyCM was prepared by PHA stimulation of mononuclear cells from blood or various tissues (spleen, lymph node, tonsil) using a method first described by Morgan et al (1976) for the production of T-cell growth factor (IL-2). At the time of initiating these studies, specific biochemical or immunochemical assays (e.g. radioimmunoassay) for the identification and measurement of the interleukins were not available and identification and quantitation of interleukin activity was dependent upon bioassay (Gillis et al 1978b). Therefore in order to define the CM used in this study conventional liquid culture bioassays for interleukin activity were used. Subsequently these CM were tested in T-cell colony forming experiments to investigate the role of soluble factors in regulating T colony formation and their ability to substitute for cellular interactions within the cultures.

MoCM was shown to contain IL-1 by demonstrating a direct mitogenic effect on thymocytes and by enhancement of PHA induced thymocyte and T-cell mitogenesis (Maizel et al 1981,

Mizel et al 1978, Mizel 1982). The type of assay used and the results obtained are similar to those used in previous studies of IL- 1, showing augmentation by murine or human monocyte conditioned media of 3H- thymidine incorporation into PHA or ConA stimulated murine thymocytes (Gery et al 1971, Gery and Waksman 1972, Calderon et al 1975, Oppenheim et al 1976, Unanue et al 1976, Lachman et al 1978) and are similar to the more recent description of enhancement of ConA stimulated human thymocyte proliferation by monocyte conditioned medium and purified IL-1 (Maizel et al 1981). Further evidence that MoCM contained IL-1 was obtained by the demonstration of enhanced IL-2 production by human T-cells and thymocytes when stimulated with MoCM in addition to PHA. These findings are similar to those of Smith et al (1980) who used a murine system and purified monocyte conditioned media to show that IL-1 enhanced IL-2 production but was inactive when tested for IL-2 activity .

IL-2 activity was demonstrated using PHA activated cultured T-cells that were unresponsive, or at least poorly responsive, to further PHA and/or IL-1 stimulation but which would proliferate in the presence of culture supernatants (LyCM) from PHA stimulated MC or T-cells . The target cells for assaying IL-2 activity were either T- CFCs or T- cells grown in suspension with PHA and LyCM. This assay system is similar to that described by other groups (Inouye et al 1980, Gootenberg et al 1981) with the exception that in the majority of experiments T-CFCs rather than conventionally

cultured T-cells were used. However as no differences could be found between T-CFCs and T-cells from cultures initiated and maintained in liquid phase, either in the cell phenotype or in their proliferative responses to PHA or CM, these assays can be considered directly comparable with those of other groups (Gillis et al 1978b, Inouye et al 1980).

Using the above assay systems, LyCM was shown to stimulate proliferation of cultured T-cells in a dose dependent manner similar to that previously described for murine IL-2 dependent T-cell lines (Gillis et al 1978b) or cultured human T-cells (Inouye et al 1980). Different batches of LyCM, especially those prepared from blood MC, varied considerably in their IL-2 activity. Spleen LyCM, prepared from spleen MC of a patient with spherocytosis, showed high levels of activity and gave near maximal stimulation at 25% concentration. In subsequent experiments on colony formation either spleen LyCM, of which approximately 500ml were prepared, or batches of blood MC CM with comparable high activity were used in order to obtain optimal and consistent T-cell stimulation.

In these experiments crude supernatants were used to supply IL-1 and IL-2 activities and it is possible that the CM contained other activities than IL-1 or IL-2. For example the possibility that LyCM contained small amounts of IL-1 in addition to IL-2 was not formally excluded as the assays for IL-1 activity, enhancement of thymocyte and T-cell

mitogenesis, are also sensitive to IL-2 and therefore do not allow distinction between CM with both IL-1 and IL-2 activity.. Results obtained using LyCM fractionated by G-100 gel filtration showed that the major T-cell stimulatory (IL-2) activity was present in fractions of around 15,000 mol.wt., corresponding to the reported mol wt of human IL-2 (Gillis et al 1980, 1982, Robb 1982), however smaller peaks of activity could be seen at higher molecular weights. These may represent high mol wt IL-2 complexes (Oppenheim et al 1980, Robb 1982) but could possibly represent other interleukins. Nevertheless in terms of bioassay it was clear that MoCM contained active IL-1 but no IL-2 and that the major T-cell stimulatory activity in LyCM both in terms of bioassay and partial biochemical characterisation corresponded to IL-2.

Enhancement of colony formation by conditioned medium

Both MoCM and LyCM enhanced T-lymphocyte colony formation as well as demonstrating interleukin activity in liquid culture. Similar results demonstrating enhanced colony formation have previously been described by various groups who have shown production of colony enhancing activity by mitogen stimulated lymphocytes and adherent cells (Claesson et al 1977b, Rosenszajn et al 1980, 1981).

When CM were assayed for T colony stimulatory activity the results obtained were found to depend on both the

composition of the lymphocyte suspension plated (e.g. blood MC, enriched T cells, thymocytes in primary culture or T-CFCs in secondary culture) and on the cell concentration in the culture wells.

With unfractionated blood MC colony formation was optimally enhanced by CM at lower cell plating concentrations ($<2x10^5$ cells/well). This finding is consistent with the hypothesis that colony formation is dependent upon release of stimulatory factors from accessory cells which as cell concentration falls fail to produce adequate amounts of growth factor(s) to drive T-CFC proliferation (Gerassi and Sachs 1978, Goube deLaForest et al 1978). In order to clarify the role of such possible cell interactions two experimental approaches were used: firstly, analysis of conditioned media requirements for colony formation using purified cell populations in cultures and secondly kinetic analysis of cell interactions in colony formation, as described by Goube deLa-Forest et al (1979b).

Colony formation by T-cells and thymocytes in primary culture

In primary culture freshly isolated T-enriched cells showed poor colony formation in comparison to MC suspensions. Colony formation was restored to normal levels by adding either MoCM or LyCM to cultures or by culturing T- cells on

feeder layers containing adherent cells. These findings are consistent with previous reports of the macrophage dependency of colony formation (Claesson et al 1977b, Rosenszajn et al 1978) and demonstrate the ability of CM with IL-1 activity to substitute for macrophages in culture. In these experiments MoCM with IL-1 activity from leukaemic monocytic cells was used since in preliminary studies I was unable to prepare active CM from normal blood adherent cells. This was probably due to the release of colony inhibitory factors from normal adherent cells (Zeevi et al 1977, Bockman and Rothschild 1979). However evidence that normal monocytes do promote T colony formation was obtained from experiments in which colony formation was enhanced when enriched T-cells were cultured on adherent cell underlayers. Further evidence for the importance of the macrophage in colony formation was provided by experiments in which blood MC were treated with the antimonocyte antibody OKM1 and complement, a procedure which depletes cell suspensions of at least 50% of the monocytes present (Poznansky 1983). In these cultures there was a 50% reduction in the mumber of colonies in response to PHA alone, but colony numbers were restored to normal in the presence of MoCM.

Treatment of blood MC with anti T-cell antibodies and complement reduced colony formation. This was most marked following treatment with the pan T cell antibody OKT3, although inhibition was also obtained with anti T-helper (OKT4) and anti T cytotoxic/suppressor (OKT8) antibodies. In

these experiments the reduction in colony formation by T-depleted cells was associated with a reduction in the ability of OKT3 and OKT4 treated cells to produce IL-2, and could be partially but not completely restored to normal levels by culturing the treated cells with LyCM. These finding suggest that the inhibition of colony formation in these experiments was the result of both removal of T-helper cells (producing IL-2) and removal of T-CFC precursors. In the case of OKT3 treated cells, although few (<5%) mature T3+ cells remained after treatment considerable numbers of colonies were formed in the presence of LyCM suggesting that T-CFC precursors might include OKT3-negative as well as OKT3-positive cells. Spitzer et al (1982) have also suggested that T-CFC precursors include not only mature OKT3-positive cells but also a population of OKT3-negative cells. These results should however probably be treated with some caution since treatment of cells with OKT3 (and complement) does not fully deplete OKT3 positive T cells from blood. I have observed that although immediately following OKT3 and complement depletion only small numbers (1-5%) of OKT3 positive cells can be detected by immunofluoresence, after overnight incubation up to 20% OKT3 positive cells can be found in cell suspensions. This suggests that loss of OKT3 positive cells may be due to shedding or endocytosis of membrane antigen as well as due to lysis of OKT3 bearing cells by complement.

Thymocytes showed poor colony formation with PHA. Colony formation was increased by the addition of MoCM to cultures

but was still low in comparison to cultures supplemented with LyCM which showed similar numbers of colonies to T-enriched blood MC suspensions. This is in contrast to previous studies of thymocyte colony formation which have shown very poor colony responses (Gelfand et al 1981). The observation that thymocyte colony formation approached that of normal T-cells in cultures containing LyCM suggests that the poor colony responses to PHA are due to a deficiency of IL-2 production by thymocytes rather than due to a lack of T-CFC precursors. The poor response of thymocytes to MoCM is consistent with the observation that thymocytes, even when stimulated with PHA and IL-1, are poor producers of IL-2 (Smith et al 1980) and with studies that have shown IL-2 producing cells constitute only a minority subpopulation of mature thymocytes whereas IL- 2 reponsive cells capable of proliferation include a large population of both immature cortical as well as mature medullary thymocytes (Wagner et al 1979).

Colony formation by T-CFCs in secondary culture

T-CFCs, in contrast to freshly isolated T-cells, in secondary agar culture proliferated to form colonies only in reponse to LyCM but not in response to PHA or MoCM alone. Identical findings were reported by Goube deLaForest et al (1979b) who suggested that the lack of colony formation in secondary culture, in the absence of LyCM, was the result of depletion of accessory cells, resposible for the production of

growth factor (colony promoting activity / CPA), from harvested T- CFCs. In the present study it was noted that occasionally a few colonies developed in secondary culture in response to PHA, although this response was less than with LyCM. This may be due to the persistence of small numbers of accessory cells in the cultures. The persistence of cooperating cells may also explain the responsiveness to PHA and MoCM observed in liquid cultures of TCFCs and the cluster formation seen in agar occurring during the 2-3 days after harvesting. The viability or functional capacity of the cooperating cells is however shortlived as is seen from the loss of responsiveness to PHA in liquid culture and the degeneration of clusters in agar after 3 days culture. The similarity in the responses to CM of T-CFCs in secondary agar culture and suspension culture with that of conventionally cultured PHA activated T cells maintained in suspension culture with LyCM suggests that sustained proliferation of T-CFCs is dependent upon IL-2. These studies of secondary colony formation do not exclude the possibility that T-cells responsive in primary culture (i.e T-CFC precursors rather than T- CFCs) may require additional or alternative growth signals to allow them to respond to IL-2.

Investigation of cell interactions in colony formation by Kinetic analysis

The role of cellular interactions in colony formation was further investigated by the application of mathematical analysis to colony formation at different cell plating concentrations. This means of analysis suggested that three independent cells interact in the formation of colonies in primary culture, in the presence of PHA. This confirms the previous finding of Goube de Laforest et al (1978, 1979a, 1979b). This analysis further suggested that in the presence of either LyCM or MoCM the number of interacting cells were reduced. When both MoCM and LyCM were added, the requirement for cell interactions appeared to be eliminated. These findings are consistent with a model of colony formation involving at least three interacting cells; firstly a colony forming cell precursor that proliferates in reponse to growth factors; secondly two growth factor producing accesory cells that are present in limiting amounts as the cell concentration in cultures fall but which can be substituted for by the addition of conditioned media with IL-1 and IL-2 activity.

Although kinetic analysis has been widely used to investigate cell interactions in the immune response (Coppleson and Michie 1966, Mosier et al 1968, Jiminez and Bloom 1972, Kondracki et al 1977, Kondracki 1979, Goube de Laforest et al 1979b, Tse et al 1980), the results of this

type of analysis should be interpreted with some caution. The kinetic theory, upon which this analysis is based (Tse et al 1980), requires that each cell should act independently and at random. It is however known that interactions between monocytes and lymphocytes are complex and involve numerous stimulatory and inhibitory interactions. For example, IL- 2 production is dependent upon the presence of IL-1 production by macrophages (Larsson et al 1980, Smith et al 1980), but IL-1 production itself is dependent on production of macrophage activating factors by activated lymphocytes (Unanue et al 1976, Unanue 1978, Mizel et al 1978). The situation is further complicated by the production of inhibitory factors by macrophages such as prostaglandins which may inhibit IL-2 production and colony formation (Bockman and Rothschild 1979, Inouye et al 1980, Rosenszajn et al 1980). A further problem in applying the kinetic theory is that the equation describing cell interactions ($\log n = b \log N + a$) is accurate only if the concentration of the cell sub-populations and overall cell concentration remains constant, or if cell interactions leading to the measured response are instantaneous. This is clearly not the case as proliferation of T-CFCs increases one sub-population and occurs over a period of days.

The problems outlined in the above paragraph may explain why experimentally derived regression coefficients (b values) do not correspond exactly to a three cell model of T-colony formation, involving one colony forming cell precursor and two accesory cells (i.e. an IL-1 producing macrophage and an

IL-2 producing T-cell). For example with MoCM experiments gave a b value of 1.4 rather than 2. A possible explanation for this is that at high cell plating concentrations MoCM may not only substitute for the IL-1 producing cell but may also, by amplification of IL-2 production, substitute partially for a relative lack of IL-2 producing cells (giving a b value of 1) whereas at low cell concentrations, although MoCM may substitute for the IL-1 producing monocyte, insufficient IL-2 producing cells are present to allow optimal IL-2 production and b values of 2 are obtained.

Growth factors and cellular requirements for colony formation

Experimental observations of the CM requirements of blood MC and purified T-cells and thymocytes in primary culture and T-CFCs in secondary culture together with kinetic analysis of colony formation clearly shows that T-cell colony formation is dependent upon cell interactions. As the present work has demonstrated that MoCM contains IL-1 and LyCM contains IL- 2, it is therefore possible to construct a model for colony formation that is analogous to current models for interleukin action on T-cells in liquid culture (Palacios 1982):

1) In the presence of PHA alone 3 cell populations are

required to interact for T-cell activation and proliferation (colony-formation); an IL-1 producing cell (monocyte), an IL-2 producing cell (T-lymphocyte) and an IL-2 responsive clonogenic cell (T-CFC precursor).

2) In the presence of PHA and IL-1 (MoCM) two cell populations are required; an IL-2 producing cell and an IL-2 responsive cell (T-CFC precursor).

3) In the presence of PHA and IL-2 (LyCM) two cell populations are required; a monocyte and an IL-2 responsive T-cell.

4) In the presence of PHA and both IL-1 and IL-2 there is a requirement for only the clonogenic IL-2 responsive T-cell.

5) In secondary culture of T-CFCs there is a requirement for IL-2 and the T-CFC alone.

A suprising finding was that in primary culture LyCM (which contained IL-2) could not by itself effectively replace the requirement for cell interactions, although LyCM could maintain proliferation of T-CFCs in secondary culture and T-lymphocytes in suspension culture for at least two to three weeks. A possible explanation for this observation is that T-CFC precursors, in contrast to cells reponding to PHA in liquid culture, may include not only mature peripheral T-

cells responsive to PHA and subsequently IL-2, but also precursors that require other stimuli to proliferate (Mossalayi et al 1982, Spitzer et al 1982). The observation that MoCM and LyCM combined allow colony formation at lower cell concentrations than with LyCM or MoCM alone suggests that IL-1 or some other factor present in MoCM may have effects other than through amplification of IL- 2 production. Possibly IL-1 present in MoCM may increase the sensitivity of T-cells to IL-2 through an effect such as induction of IL-2 receptors although at present there is no direct experimental evidence for this hypothesis. An alternative explanation for the synergy of MoCM with LyCM in promoting colony formation is that some stimulatory factor(s) other than IL-1 is present in MoCM. A recently defined interleukin, named IL-3, has been described that induces differentiation of immature T- cells into mature IL-2 producing or responsive cells (Ihle et al 1982). One source of IL-3 is the myelo- monocytic murine cell line WEHI-3 (Ihle et al 1982). It would be interesting to determine whether the MoCM used in this study and derived from a human monocytic leukaemia produced a factor with similar activity.

A consistent observation in all experiments was that below a critical cell concentration no colony formation occured, even in cultures containing both MoCM and LyCM. This finding suggests that direct cell-cell interactions in addition to humoral interactions also play an important role in the induction of colony formation. Recent studies have

shown that both production of IL-2 and acquisition of responsiveness to IL-2 depends upon interaction of T-cells with HLA-DR determinants expressed on accesory cells, such as the macrophage (Palacios 1982, Hunig 1983, Larsson and Couthino 1984). These findings may account for the observations that LyCM (IL-2) alone is unable to replace cell interaction requirements in primary culture, and also may explain the inability to induce colony formation below critical cell concentrations where the probability of cell-cell contact is low.

Recently Klein et al (1982) have proposed a 3 cell model for colony formation similar to that outlined above. They have however questioned the role of IL-2 in T colony formation and proposed that the cooperating cells in colony formation include not only IL2 producing T-cells but also non adherent non-T cells, that release a colony stimulating factor (colony promoting activity or CPA) following mitogenic stimulation. Evidence for this is based upon the observation that high levels of CPA can be found in the supernatants of mitogen stimulated B-cells (purified by anti-Ig affinity chromatography). In subsequent studies this group compared IL-2 production with CPA production by various MC subsets (Mossalayi et al 1982). These studies showed no CPA or IL-2 production by purified E+ T cells or adherent cells alone but did show that a mixture of T cells plus adherent cells produced both IL-2 and CPA. Suspensions of E negative, non adherent cells (i.e. B-cells and null cells), still produced

CPA but did not produce IL-2. CPA production by E negative cells was not affected by lysis of OKT3+ cells but was abolished by treatment with the anti T-cell antibody A50. This "B-cell factor" may therefore have been produced by that small numbers of contaminating T-cells (perhaps E-, OKT3-, A50+) and monocytes present in the suspensions that were used to prepare CM. Further it is known that B-cells may indirectly enhance T-cell proliferation by enhancing IL-2 production (Inouye et al 1980) through T-B cell interactions mediated by HLA-DR determinants expressed by B-cells that render T-cells more sensitive to the effects of both IL-1 and IL-2 (Palacios 1982). This question of whether B-cells stimulate colony formation through release of specific B-cell derived growth factors or indirectly through products such as membrane associated HLA-DR antigens can be adequately resolved only through taking stringent precautions to ensure the purity of the B-cell suspensions or, perhaps best, by using cloned normal B-cells or B-cell hybridomas to prepare CM.

Conclusions

These studies have shown that the colony formation can be successfully used to analyse cell interactions in T-lymphocyte proliferation and have clearly demonstrated that T-cell colony formation is dependent upon interactions between T-CFC precursors and accessory cells. Colony formation is enhanced by MoCM and LyCM. These CM can substitute, at least

partially, for accesory cell/T-CFC interactions in culture. As these CM contain IL-1 and IL-2 activity, it is probable that colony formation is regulated by IL-1 and IL-2, either singly or in combination, however the possibility that other less well defined factors also play a role has not been excluded . It is possible that T-CFC precursors are heterogeneous and may be regulated by factors other than IL-1 and IL-2 , with different factors acting on cells at different stages of differentiation. Further clarification of the interrelationship of the interleukins and T-cell colony stimulating factors and the identification of distinct colony stimulating factors requires further study using biochemically characterised and purified media.

Despite the limitations imposed by the use of incompletely defined growth factors in colony forming assays, this experimental system provides a method for the definition of abnormalities in T-cell function. Subsequent chapters describe how colony formation may be used not only to detect changes in T-cell function occuring in disease but also to determine whether these changes are the result of abnormalities in growth factor mediated cell interactions.

CHAPTER 3

COLONY FORMATION IN LYMPHOPROLIFERATIVE DISEASE

SUMMARY

In this chapter I describe investigations of T-cell colony formation in two forms of lymphoproliferative disorder: T-cell chronic lymphocytic leukaemia (T-CLL) and Hodgkin´s disease.

In the first part of this chapter (chapter 3.1) studies of colony formation by leukaemic T-cells are described. Colony formation by T-CLL cells was shown to be dependent on growth factors in a similar manner to colony formation by normal T-lymphocytes. It was also demonstrated that leukaemic cells could not only respond to growth factors but were also able to produce normal or increased amounts of interleukin 2.

In Hodgkin´s disease T-lymphocyte proliferation is frequently depressed. The mechanisms involved in this defect have however not been clearly defined. In these investigations of colony formation in Hodgkin´s disease (chapter 3.2) I have demonstrated that T-cells from these patients show impaired colony formation. Interleukin 2 production was also shown to be decreased. Evidence is presented that suggests that the reduction in colony formation may be the result of alterations in blood T- cell subsets and in some cases secondary to impaired IL-2 production.

CHAPTER 3, part 1

COLONY FORMATION BY LEUKAEMIC HUMAN T-CELLS

SUMMARY

PHA induced colony formation and interleukin 2 (IL-2) production were studied in four patients with chronic T-cell leukaemia. In three of the cases the leukaemic cells showed an OKT4+/T- helper phenotype and in one case cells showed an OKT8+/T- cytotoxic/suppressor phenotype. The cases of T-helper cell leukaemia showed colony formation that was comparable to normal blood T cells and was not dependent on the addition of conditioned medium, containing IL-2 activity, to cultures. In contrast the T-suppressor cell leukaemia formed colonies only when cultures were supplemented with IL-2 containing medium.

When IL-2 production by PHA stimulated cells was measured, culture supernatants from the three T-helper cell leukaemias all showed normal or high levels of activity, when compared to supernatants from normal blood mononuclear cells, The T suppressor cell leukaemia showed no evidence of IL-2 production. Culture supernatant from one case of T-helper cell leukaemia was fractionated by passage through G-100 Sephadex and was shown to give a similar pattern of elution of IL-2 activity as culture supernatants from normal mononuclear cells.

INTRODUCTION

T-cell lymphocytic leukaemia is a rare form of chronic lymphocytic leukaemia (CLL) in which the leukaemic cells have been shown to express differentiation markers characteristic of mature T-lymphocytes (Pandolfi et al 1982, Catovsky 1984). The neoplastic cells in these cases usually express the T3 antigen (common to all mature T-lymphocytes) and either the T4 antigen (T- helper/inducer) or T8 antigen (T-cytotoxic/ suppressor) and can therefore be subclassified as T-helper/inducer or T- cytotoxic/suppressor cell leukaemias (Reinherz and Schlossman 1981, Foon et al 1982), although less commonly unusual phenotypes such as T3 with both T4 and T8 have been reported (Foa et al 1982, Vyth et al 1982).

Recently functional studies of T-cell CLL have shown that in some cases these cells proliferate in response to mitogens in vitro (Gramatzki et al 1982, Pandolfi et al 1982) and can be induced to form colonies (Foa et al 1982). It has also been shown that some cases of T- cell CLL produce interleukin 2 (IL-2) following stimulation by mitogens such as PHA (Friedman et al 1982) or TPA (Vyth et al 1982), and may also be able to respond to IL-2 (Devries et al 1981). These findings suggest that the proliferation of leukaemic T-cells may be regulated, at least in part, by growth factors, such as the interleukins, in a similar manner to normal T- lymphocytes.

In this study colony formation and IL-2 production by

neoplastic T-cells, from three patients with T-helper/inducer CLL and one patient with T-cytotoxic/suppressor CLL, were investigated in order to determine to what extent these leukaemic cells produce or respond to the growth factors that modulate normal T-cell proliferation. Responsiveness to interleukin 1 (IL-1) and interleukin 2 (IL-2) was determined by measuring colony formation in cultures supplemented with conditioned media containing IL-1 (MoCM) or IL-2 (LyCM). Production of IL-2 following PHA stimulation of the leukaemic cells was also measured.

MATERIALS AND METHODS

Isolation of blood mononuclear cells (MC:) Blood MC from normal individuals and patients with T-cell leukaemia were isolated from venous blood by centrifugation over Ficoll-Hypaque as previously described (chapter 1). MC from the plasma-Ficoll interface were washed twice in RPMI 1640 and resuspended at the required cell concentration for subsequent cell phenotyping or culture.

Cell phenotyping: Rosetting with sheep red blood cells (E-rosettes) and mouse red blood cells (MRBC-rosettes) was carried out as previously described (see chapter 1. Stockdill et al 1983).Lymphocyte subsets were identified using the monoclonal anti T-cell antibodies; OKT3, OKT4, OKT8 (Ortho Diagnostics Ltd), and the anti HLA-DR antibody DA6.231 (Guy et al 1983). Positive cells were detected using an indirect immunofluorescence assay (chapter 1).Cytochemical staining for acid phosphatase and non specific esterase was performed on blood films and cytospin preparations.

Colony Formation: Colony formation was assesed as previously described. Cells were suspended at $5x10^6$ cells/ml in 0.33% agar in RPMI 1640 containing 15% heat inactivated human serum (HIHS). Forty microlitres of this suspension ($2x10^5$ cells) was pipetted onto underlayers of 0.2ml of 0.5% agar containing 15% HIHS, 1% PHA and 0.5% autologous red blood cells. Cultures were also carried out on underlayers

supplemented with 25%v/v spleen lymphocyte conditioned medium (LyCM) or 15%v/v monocyte conditioned medium (MoCM). Cultures were carried out in 17mm diameter wells in Linbro 24 well dishes. Colonies (aggregates of more than 40 cells) were counted after 5-6 days incubation at 37°C in a fully humidified atmosphere of 5% CO2 in air.
Colony cells were harvested from cultures for immunophenotyping as previously described (chapter 1).

IL-2 production: MC from normal individuals and leukaemic patients were cultured at $1x10^6$cells/ml in RPMI 1640, with 1% PHA, 1% HIHS and 1microgram/ml indomethacin (Sigma laboratories) (Inouye et al 1980). Supernatants were harvested after 48 hours incubation and stored at -20°C until assayed for IL-2 activity.Assays for IL-2 activity in supernatants were carried out as previously described in chapter 2. Target cells for IL- 2 assays were T- colony cells harvested from primary cultures after 6-7 days incubation (chapter2).

Biochemical characterisation of IL-2 by gel filtration:
The T-cell stimulatory factor(s) in normal and leukaemic cell culture supernatants were partially characterised and compared using Sephadex G-100 gel filtration. Gel filtration was carried out as described by Gillis et al (1980) with minor modifications. One hundred ml volumes of supernatant were precipitated using 85% saturated (NH4)2SO4. The precipitate was collected, dialysed against phophate buffered saline (PBS)

(pH 7.2), and reconstituted in 2.0ml of PBS. This was then passed through a 90x5cm column containing Sephadex G-100 that had previously been equilibrated with PBS and calibrated using blue dextran, bovine serum albumin, ovalbumin, chymotrypsinogen A, and cytochrome C as molecular weight markers. Fractions were collected in 8.0ml aliquots and stored at $4^{o}C$. IL-2 activity in fractions was measured by culturing T-colony cells with fractions at 50% concentration, as desribed above (fig 3.1).

RESULTS

Patients studied: Four patients with chronic T cell leukaemia were studied. In cases 1-3 blood MC showed a T-helper phenotype (OKT3+,OKT4+) and in case 4 a T-cytotoxic/suppressor phenotype (OKT3+,OKT8+). The leukaemic cells also showed cytochemical staining typical of T-cells with focal acid phosphatase and non specific esterase staining of cells. All cases were negative for Tdt and did not express B-cell markers (HLA-DR, mouse red blood cell receptor, surface Ig). Morphologically cases 2 and 3 were classified as lymphocytic leukaemias and cases 1 and 4 as prolymphocytic leukaemias (table 3.1).

Colony formation: Colony formation was measured on underlayers containing either PHA alone or supplemented with MoCM or LyCM. The three cases of T- helper (OKT4+) cell leukaemia showed colony formation that was comparable to that of normal blood T-cells. Colony formation in these cases was enhanced by both MoCM and LyCM. In contrast the T-cytotoxic/suppressor (OKT8+) cell leukaemia (case 4) showed colony formation only in cultures supplemented with LyCM (table 3.2). In this case colonies were small (40-50 cells) and showed early degeneration (5 days), whereas colonies in cases 1-3 were large (>100 cells) and continued to grow without evidence of degeneration until day 6-7 of cultures.

TABLE 3.1 CELL PHENOTYPE IN FOUR CASES OF CHRONIC T CELL LEUKAEMIA

				% positive mononuclear cells						
Patient	Age/Sex	WCC	%lyms	OKT3	OKT4	OKT8	HLA-DR	E	MRBC	Diagnosis
1.	73 M	213	98	98	97	5	1.5	85	2	T-helper prolymphocytic leukaemia
2.	82 F	51.5	97	96.5	100	0.5	-	77.5	0.5	T-helper lymphocytic leukaemia
3.	65 F	47	85	81	100	1.0	1.0	17	1.0	T-helper lymphocytic leukaemia
4.	49 M	15	95	94	25*	88.5	1.0	79.5	6.5	T-suppressor/cytotoxic prolymphocytic leukaemia
Normal range				46-70	33-64	16-32	15-30	43-68	0-5	

*(weak reaction)

All cases were Tdt negative. Cytochemical staining showed strong focal acid phosphatase and non-specific esterase activity in cases 1-3 and strong acid phosphatase but weak non-specific esterase activity in case 4.

Colony cells harvested from cultures were morphologically large transformed cells similar in appearance to normal T-CFCs. Leukaemic T-CFCs showed a marked predominance of either OKT4+ or OKT8+ cells in contrast to normal T-CFCs which contaioned a mixture of OKT4+ and OKT8+ cells (table 3.3).

TABLE 3.2 COLONY FORMATION BY NORMAL AND LEUKAEMIC T CELLS

Culture underlayer:	Colonies		
	PHA	PHA+MoCM	PHA+LyCM
Patient 1 (OKT4+)	32±4	225±15	252±13
2 ..	12±7	85±14	128±27
3 ..	122±18	253±16	253±10
4 (OKT8+)	0	0	76±8
Normal MC (4)	154±33	171±35	175±28
.. T cells (4)	20±16	198±98	216±82

Figures for patients 1-4 are the mean values ± 1 S.D. of triplicate cultures in single experiments.
Figures for normal MC and T cells are mean values ± 1 S.D. of four experiments. Blood MC contained 47-68% T cells (E-rosetting) and 10-13% monocytes (non specific esterase positive cells). T cells purified by rosetting with sheep RBC contained 93-98% T cells and less than 1% monocytes.

TABLE 3.3 PHENOTYPE OF COLONY CELLS

Patient	% positive cells			
	OKT3	OKT4	OKT8	E-rosettes
1	97	93	0	99.5
2	93	92	0	99.5
3	72	85	3	93
4	not tested	10	43	not tested

IL-2 production: Supernatants from PHA stimulated cultures of leukaemic cells were tested for IL-2 activity by assessing their ability to stimulate proliferation of normal T-CFCs. Supernatants from the three cases of OKT4+ leukaemia all showed normal or high levels of IL-2 activity, when compared with supernatants from normal blood MC. The OKT8+ leukaemia showed no evidence of IL-2 production (table 3.4).

Gel-filtration of normal and leukaemic cell culture supernatants: The IL-2 activity in both normal (spleen MC LyCM) and leukaemic (case 3) cell culture supernatants showed an almost identical pattern of elution from G-100 sephadex. The major peak of activity was present in fractions of about 15,000 mol.wt, with a smaller peak of activity in a high mol.wt fraction eluting after blue dextran (fig 3.1).

TABLE 3.4 INTERLEUKIN 2 PRODUCTION BY NORMAL AND LEUKAEMIC CELLS

% Concentrations of Conditioned Medium	IL-2 activity (cpm) 50%	25%	12%	6%
Case 1: Ex 1	11,200	6,856	4,150	1,755
Ex 2	11,385	4,516	4,916	2,042
Ex 3	-	8,854	6,999	3,098
Ex 4	10,673	8,652	4,299	2,451
Case 2: Ex 1	12,508	13,407	11,492	9,353
Ex 3	12,837	11,634	9,250	8,107
Case 3: Ex 2	13,240	4,803	13,555	10,538
Ex 4	18,843	16,821	15,103	9,522
Case 4: Ex 2	1,027	1,645	984	286
Ex 4	735	445	496	286
Normal: Ex 1 blood MC	9,192 ± 2,887	7,123 ± 4,108	4,771 ± 3,057	2,240 ± 1,184
Spleen MC: Ex 1 (LyCM)	11,573	5,965	3,821	3,120
PHA control: Ex 1	1,400	482	906	1,105
Ex 2	1,035	1,830	582	705
Ex 3	1,547	748	682	837
Ex 4	115	-	-	-

Conditioned media from PHA stimulated MC were prepared and tested for IL-2 activity as described in materials and methods. Figures for normal MC are the mean value ± 1 SD of IL-2 activity in conditioned medium from eight different normal individuals. In each experiment a PHA control solution (1% PHA in RPMI 1640) was tested at the given dilutions. Responses to RPMI 1640 alone were 330 cpm (Expt 1), 687 cpm (Expt 2), 637 cpm (Expt 3) and 225 cpm (Expt 4).

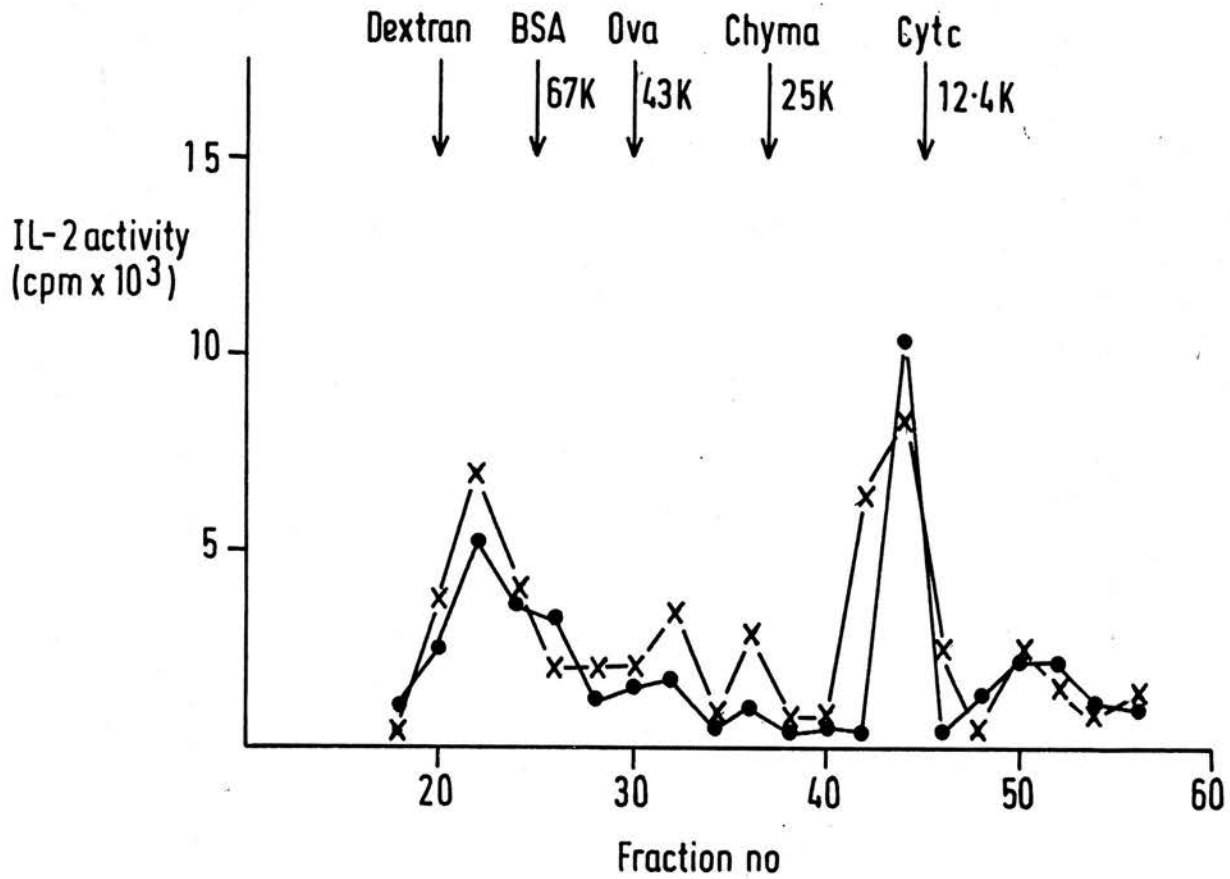

FIGURE 3.1 SEPHADEX G-100 FRACTIONATION OF CONDITIONED MEDIUM FROM A PATIENT WITH T-CLL

Fractions were assayed for IL-2 activity by culturing T-CFCs with 50% v/v test medium as described in methods.
T-CLL conditioned medium =
Normal MC conditioned medium =
Responses of T-CFCs in control cultures were; medium alone 1,236 c.p.m., PHA 0.5% v/v 2,496 c.p.m., unfractionated T-CLL conditioned medium 25,532 c.p.m., unfractionated normal MC conditioned medium 9,654 c.p.m..

DISCUSSION

This study shows that colony formation can be induced in leukaemic T- cells by PHA and shows a similar growth factor dependency to that of normal T- cells. T- helper (OKT4+) cell leukaemias formed similar numbers of colonies to normal T-cells and showed similar enhancement of colony formation in the presence of MoCM (IL-1) and LyCM (IL-2) . In contrast OKT8+ leukaemic cells formed fewer colonies than normal T cells or OKT4+ leukaemic cells and were completely dependent upon the addition of IL-2 containing CM to cultures. In an earlier investigation of T- cell colony formation in T-cell leukaemia, similar results were obtained by Foa et al (1982) who demonstrated near normal colony formation by OKT4+ leukaemias, but markedly subnormal colony formation in OKT8+ leukaemias, although in this study (Foa et al 1982) growth factor requirements for colony formation were not investigated.

The differences in the culture requirements of OKT4+ and OKT8+ leukaemic cells can be explained by differences in the ability of leukaemic cells to produce IL-2, since OKT4+ cells produced IL-2 in response to PHA whereas OKT8+ cells did not. Claesson et al (1983) have reported similar findings using FACS purified T-helper (Leu3+/T4+) and T-cytotoxic/suppressor (Leu2+/T8+) normal lymphocytes. In this study they showed that whereas T-helper cells formed colonies in response to PHA alone, T-cytotoxic/suppressor cells required the addition of

IL-2 to cultures to allow colony formation (Claesson et al 1983). These findings are consistent with the observation that IL-2 production by normal T-cells is a function of the OKT4+ T-cell subset (Reinherz et al 1980b, Palacios 1982), and with previous observations that OKT4+ T-cell leukaemias can be induced to produce IL-2 following mitogenic stimulation (Friedman et al 1982, Gramatzki et al 1982, Vyth et al 1982). There are no reports in the literature on IL-2 production by OKT8+ T-cell leukaemias.

The role of growth factors such as IL-1 and IL-2 in maintaining proliferation of leukaemic T-cells in vivo is unknown. In this study no IL-2 production was detected without PHA stimulation, further the freshly isolated leukaemic cells did not respond to purified, mitogen free IL-2 (obtained from R.C.Gallo), although they could be maintained in proliferation in liquid culture for 3-6 weeks by purified IL-2 following initial PHA or TPA stimulation (D.Onions, personal communication). It has however been shown that neoplastic T-cells from some human T-cell tumours can not only be maintained by culture in IL-2 but may, in some cases, also spontaneously produce IL-2 (Gootenberg et al 1981). There is therefore a possibility that autostimulation, by IL-2 producing/resposive cells may play a role in neoplastic T-cell proliferation in vivo.

At present the clinical significance of differences in function of phenotypically mature T-cell neoplasms is unknown.

However it is possible that once these differences have been fully defined they may be exploited as either prognostic indicators or targets for the control of cell proliferation.

CHAPTER 3 part 2

COLONY FORMATION IN HODGKIN'S DISEASE

SUMMARY

T-cell colony formation and interleukin 2 (IL-2) production were studied in thirty eight patients with Hodgkin's disease. Both colony formation and IL-2 production were found to be significantly lower than normal. Studies of T lymphocyte subsets in these patients showed abnormal T-helper:T-suppressor (OKT4:OKT8) cell ratios in some patients. A positive correlation was observed between T-helper cell numbers and colony formation and a negative correlation was observed between T-suppresor cell numbers and colony formation, suggesting that abnormal colony formation may result from alterations in blood T-cell subsets. Although no statistically significant correlation could be made between colony formation and IL-2 production, preliminary studies have shown that subnormal colony formation may in some cases be restored to normal by the addition of IL-2 containing conditioned medium to cultures.

INTRODUCTION

Hodgkin's disease (HD) is a common form of malignant lymphoma in which there is frequently marked depression of cell mediated immunity both in vivo and in vitro (Winkelstein et al 1974, Twomey et al 1975, Case et al 1976, Goodwin et al 1977, Hillinger and Herzig 1978). At present the precise mechanisms underlying this impairment of cellular immunity are not clearly defined, although a number of explanations have been proposed. These include a reduction in blood T-cell numbers (Bobrove et al 1975, Case et al 1976) or increased suppressor cell activity (Hillinger and Herzig 1978). However although some investigators have found a reduction in blood T cell numbers (Bobrove et al 1975, Case et al 1976, Posner et al 1981) other groups have found no abnormality in T cells (Schulhof et al 1981, Dorreen et al 1983). There is also no agreement as to whether the proportion of T suppressor cells are increased (Romagni et al 1978) or normal (Posner et al 1981, Dorreen et al 1983), at least when rosetting techniques (Tg, Tm rosettes) or monoclonal antibodies (anti T4- helper and anti T8- suppressor) are used to define suppressor T-cells. Functional studies have however shown that there is suppression of mixed leucocyte culture responses by both lymphocytes and monocytes (Twomey et al 1975, Hillinger and Herzig 1978) and increased T cell suppressor activity is demonstrable following mitogenic stimulation of blood lymphocytes (Schulhof et al 1981). Evidence has also been presented showing that impaired PHA responsiveness may be

attributable to increased secretion of prostaglandins (PGE) by blood monocytes (Goodwin et al 1977).

Several groups have studied T-cell colony formation in Hodgkin´s disease and shown that colony formation is reduced (Dao et al 1978, Bockman 1980, Schulhof et al 1981). Bockman (1980) has suggested that the reduction in colony formation results from suppression of T-cell proliferation by increased monocyte prostaglandin production. However the possibility that other mechanisms may be involved, such as a reduction of colony forming precursors or an increase in T-suppressor cells, have not been excluded (Douer and Sachs 1979).

The aim of the present investigation was to determine whether the reduction in colony formation, reported in Hodgkin´s disease, was associated with abnormalities in blood T-cell numbers or secondary to abnormalities of growth factor production by accesory cells that modulate proliferation of colony forming cells (Rosenszajn et al 1981, Klein et al 1982, this thesis chapter 2). In order to do this colony formation and interleukin 2 (IL2) production by blood mononuclear cells were measured in patients with Hodgkin´s disease.

MATERIALS AND METHODS

Patients: Thirty eight patients with histologically confirmed diagnoses of Hodgkin's disease and thirty normal individuals were studied. Patients studied were either newly diagnosed, untreated cases or cases that were in remission and had not received chemotherapy or radiotherapy in the previous 6 weeks. Details of histological type, stage, and individual results of experimental studies are given in table 3.5.

Blood MC: MC were isolated from anticoagulated (containing 0.2% EDTA) venous blood samples by centrifugation over Ficoll-Hypaque. T cell percentages and subsets in MC suspensions were determined by rosetting and indirect immunofluorescent staining with monoclonal antibodies as desribed in chapter 1.

Colony formation: Colony forming assays were carried out in cultures containing PHA and autologous RBC underlayers, as described in chapter 1. Colony formation was also measured in cultures supplemented with indomethacin (Sigma) at 1 microgram/ml in the underlayer. Blood MC were plated in the culture overlayer at $1x10^5$ cells/well and in some cases also at $2x10^5$ cells/well.

Interleukin 2 production by blood MC: Blood MC were suspended at $1x10^6$ cells/ml in RPMI 1640 containing 1% PHA and 1% pooled normal heat inactivated human serum (with and

without indomethacin at 1 microgram/ml). Cells were cultured in 10ml plastic centrifuge tubes at 37°C in 5% CO2 in air for 48 hours. Culture supernatants were harvested following centrifugation (500g for 15 minutes) and were stored at -20°C until assayed.

Measurement of IL-2 activity: Supernatants were assayed for IL-2 activity as described in chapters 2 and 3. In preliminary experiments culture supernatants from both normal individuals and HD patients were found to give maximum stimulation of target T-cells at 50% v/v with progressive reduction in activity at lower concentrations (see FIG 3.3). All supernatants were therefore subsequently tested at 50% v/v and IL2 activity was expressed as a function of the response (3H-thymidine incorporation in counts per minute - cpm) to a standard batch of IL-2 containing medium prepared from blood MC of a patient with T-CLL:

$$\text{IL-2 units} = 100 \times \frac{\text{activity of test supernatant 50\% v/v (cpm)}}{\text{activity of T-CLL supernatant 50\%v/v (cpm)}}$$

In all experiments responses of target cultured T-cells to T-CLL supernatants were between 10-20 times that of responses to medium alone or PHA (0.5%) in control cultures.

Blood T-cells:- MC isolated from the blood of patients with HD showed no overall difference from normals in the percentage of E-rosetting cells although there was wide variation in individual results (TABLE 3.5, TABLE 3.6). However when total T-cells were enumerated using OKT3 there was found to be a small but significant reduction in the percentage of OKT3 positive blood MC. This appeared to be mainly the result of a decrease in the percentage of OKT4 positive cells with a resulting significant reduction in the T helper:suppressor (OKT4:OKT8) cell ratio (table 3.6), although again there was wide variation between individual patients (Appendix 1).No correlation was found between total T- cells or T-cell subsets and disease stage, histological type or prior treatment.

Colony formation in Hodgkin's disease :- Colony formation by blood MC from HD patients was variable. More than half the patients studied showed subnormal colony formation and overall colony formation was significantly depressed when compared to normal individuals (TABLE 3.7, FIG 3.2). In a small number of patients colony formation, that was subnormal with PHA, was within normal limits when cells were cultured with indomethacin (FIG 3.2).

TABLE 3.5A EXPERIMENTAL DATA IN HODGKIN'S DISEASE PATIENTS

1A. CLINICAL DATA AND BLOOD T-CELLS

Case	Diag	Stage	Treatment status	% positive blood MC E-rosette	OKT3	OKT4	OKT8
1	LP	2A	R	27			
2	LP	3A	R	30			
3	LP	2A	D	26	22	10	11
4	LP	2A	R	83			
5	LP	2B	D	50			
6	LP	3A	R		68	20	18
7	LP	not known	D		47	13	24
8	LP	2	D		82	21	60
9	MC	1A	R	41			
10	MC	1A	D	64	25	7	13
11	MC	3A	R	68			
12	MC	4B	R	17			
13	MC	2B	D	53			
14	MC	2A	D	55	45	19	24
15	MC	2A	D	40	60	42	16
16	MC	3B	R	34			
17	MC	1A	R	80			
18	MC	4	D		55	31	27
19	MC	3B	R	42			
20	MC	3	D	41			
21	MC	2	D	36	11	7	6

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TABLE 3.5A continued

22	MC	3B	D		62	14	42
23	NS	1A	R	30	26	11	15
24	NS	3	R	40			
25	NS	2A	R	81			
26	NS	4A	R	81			
27	NS	3A	D	62	34	16	16.5
28	NS	2A	D	50	58	32	22
29	NS	3A	R	51	51	35	9
30	NS	3B	R	58			
31	NS	3B	D	70			
32	NS	3A	R	72	74	34	43
33	NS	1A	R	75			
34	NS	3B	R	56			
35	NS	2A	D	44	41	21	8
36	NS	3A	D		55	35	15
37	LD	4B	D	80			
38	LD	4B	D		58	25	41

Diagnoses were made by histological examination of lymph nodes and classified using the Rye classification (Lukes et al 1967). Patients were staged according to the Ann-Arbor staging system (Carbone et al 1971). Patients were either newly diagnosed (D) or in remission (R) following treatment. LP=lymphocyte predominant,MC=mixed cellularity,NS=nodular sclerosis,LD=lymphocyte depleted.

TABLE 3.5B COLONY FORMATION AND INTERLEUKIN 2 PRODUCTION

Case	Diag	Stage	Colony formation $1x10^5$ cells/well		$2x10^5$ cells/well		IL-2 activity	
			PHA	PHA+ind	PHA	PHA+ind	PHA	PHA+ind
1	LP	2A	2	0	63	62	4	22
2	LP	3A	7	69	66	230		
3	LP	2A	66	107	79	186		
4	LP	2A	0	6	99	131		
5	LP	2B	73	76	131	147		
6	LP	3A	0	0			6	9
7	LP	not known					5	6.5
8	LP	2					18	14.5
9	MC	1A	3	3	5	6		
10	MC	1A	9	67	32	139	8	15
11	MC	3A	0	0	34	84		
12	MC	4B	15	30	38	124		
13	MC	2B	3	25	44	104	3	3
14	MC	2A	2	2	79	117		
15	MC	2A	52	81	82	157		
16	MC	3B	10	105	170	232		
17	MC	1A	0	0				
18	MC	4	11	15			5	6.5
19	MC	3B	20	55				
20	MC	3					3.5	5
21	MC	2					6.5	
22	MC	3B					9	10
23	NS	1A	11	62	36	121	2	8

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TABLE 3.5B continued

24	NS	3	48	74	69	147		
25	NS	2A	50	150	81	241		
26	NS	4A	0	0			53.5	127.5
27	NS	3A	7	7			26.5	172
28	NS	2A	43	63			11	49
29	NS	3A	46	78			4	4
30	NS	3B	31	94				
31	NS	3B	79	105			87	133
32	NS	3A	59	106			152	88
33	NS	1A	99	142				
34	NS	3B	83	208				
35	NS	2A					4.5	3
36	NS	3A					6.5	6
37	LD	4B	17	23				
38	LD	4B					17	28

Figures for colony formation and interleukin 2 avtivity are the mean values of triplicate cultures. Colony formation and production of supernatants from blood MC were carried out as described in materials and methods.

TABLE 3.6 BLOOD T CELLS IN HODGKIN'S DISEASE

	% Positive cells ± SEM		
	normals	Hodgkin's disease	P
E-rosettes	55.0±2.4	52.8±3.4 (31)	NS
OKT3	57.3±2.7	48.7±4.4	=.05
OKT4	40.9±2.5	22.6±2.6	<.001
OKT8	22.2±1.3	22.7±3.4	NS
T4:T8 ratio	1.99±0.19	1.29±0.22	<.01

Figures are the results of studies on 17 normal individuals and 18 patients with HD, except for E-rosetting which was measured in 31 patients. Significance testing was by the Mann Whitney U test.

TABLE 3.7 COLONY FORMATION IN HODGKIN´S DISEASE

	colonies ± SEM		
	normals	Hodgkin´s disease	P
MC cultured at $1x10^5$ cells/well			
+PHA	91.7±9.8 (19)	29.7±5.4 (30)	<.001
+PHA + Indomethacin	126.8±12.1	58.4±9.8	<.025
MC cultured at $2x10^5$ cells/well			
+PHA	130.6±15.1 (19)	69.2±10.2 (16)	<.001
+PHA + Indomethacin	177.3±15.9	139.2±15.7	<.01

Figures in parentheses show the number of cases studied. P values calculated by the Mann Whitney U test.

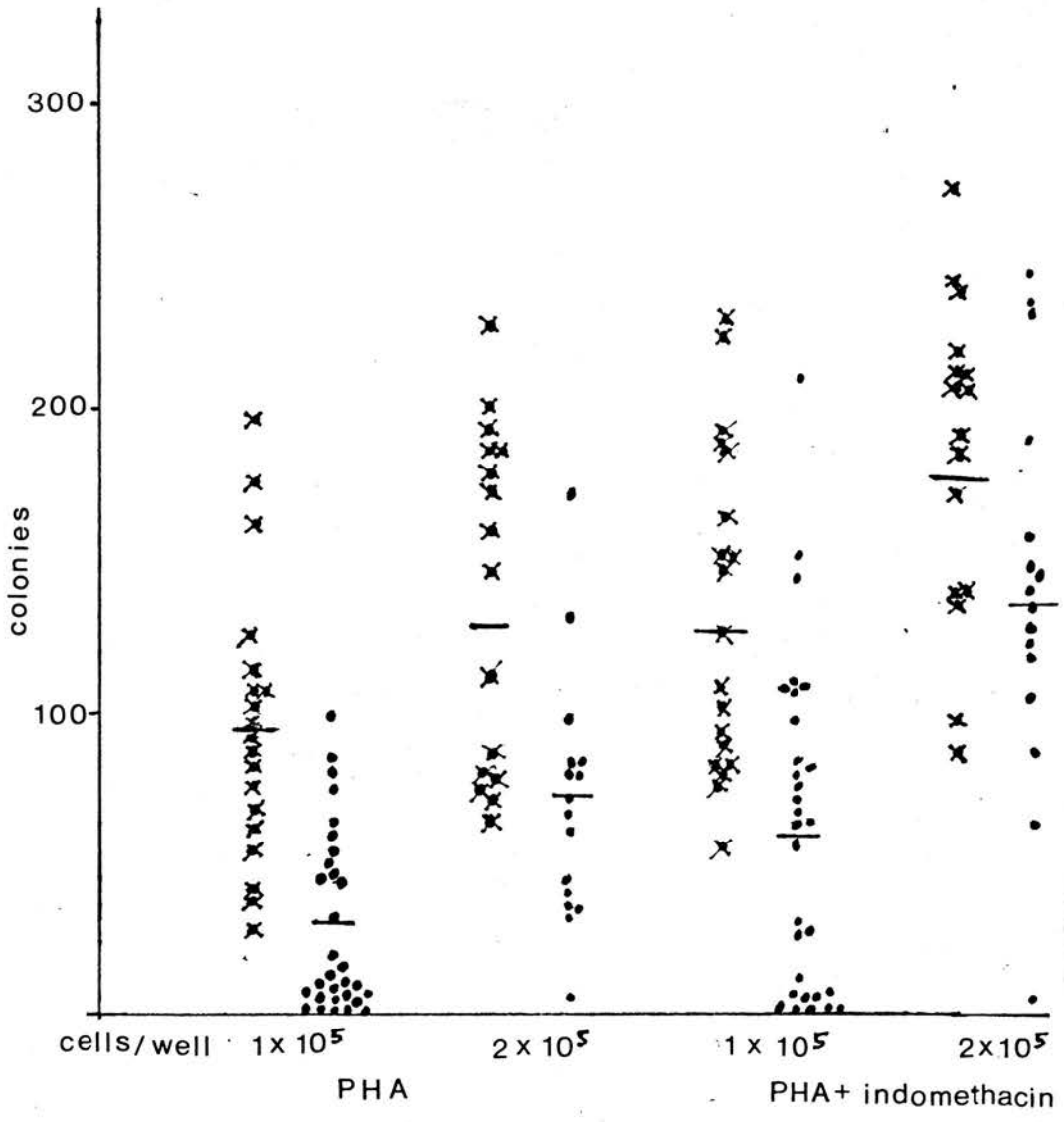

FIGURE 3.2 COLONY FORMATION IN HODGKIN'S DISEASE
Each point represents the mean value of triplicate cultures.

Normal ✕ Hodgkins Disease •

There was no correlation between colony formation and the percentage of T-cells (E-rosette or OKT3 positive cells) (P= -0.5 Kendall rank correlation) present in cell suspensions. There was however a weak positive correlation between the percentage of OKT4 positive cells and the number of colonies (P= .06) and a negative correlation with the percentage of OKT8 positive cells (P=-.07). There was no correlation between stage or treatment history but patients with nodular sclerosing type HD formed significantly more colonies than patients with mixed cellularity type disease (P<.01, Mann Whitney U test). Statistically significant differences between lymphocyte predominant and mixed cellularity or nodular sclerosing could not be demonstrated (TABLE 3.8).

TABLE 3.8 COLONY FORMATION IN HODGKIN'S DISEASE AND HISTOLOGICAL TYPE.

Histological type	Colonies at 1 x 10^5 cells/well	
	PHA	PHA+indomethacin
Lymphocyte predominant (6)	24.7±34.9	43.0±46.7
Nodular sclerosis (12)	46.3±30.9	90.7±58.5 P<.01
Mixed cellularity (11)	11.4±14.9	34.8±36.8
Lymphocyte depleted (1)	17.0	23.0

Figures for colonies are the mean values ± 1S.D.. The number of cases studied are shown in parentheses. P values were calculated using the Mann Whitney U test.

IL-2 production in Hodgkin's disease:- IL-2 activity in supernatants from HD blood MC was significantly less than that in normal blood MC culture supernatants (TABLE 3.9). Addition of indomethacin to cell cultures during stimulation with PHA increased IL-2 activity of culture supernatants of both normals and HD patients (TABLE 3.9, FIG 3.3, FIG 3.4), but only increased IL-2 activity to within the normal range in one patient that showed subnormal activity with PHA stimulation alone .

TABLE 3.9 IL-2 PRODUCTION IN HODGKIN'S DISEASE

	IL-2 units±SEM				
	normals		HD		P
supernatants from MC stimulated with:					
PHA	35.3±4.0	(17)	18.1±5.8	(20)	<.001
PHA+indomethacin	59.2±6.4	(17)	37.1±18.6	(19)	<.01

Figures in parentheses are the number of samples tested. IL-2 activity was determined as described in methods. P values were calculated by the Mann Whitney U test.

No correlation could be made between IL-2 production and T-cell numbers or subsets. There was no significant correlation between colony formation and IL-2 production. As with colony formation there was no correlation with disease stage or prior treatment but again patients with nodular sclerosing HD showed significantly higher levels of IL-2 (P<.001) than patients with mixed cellularity disease.

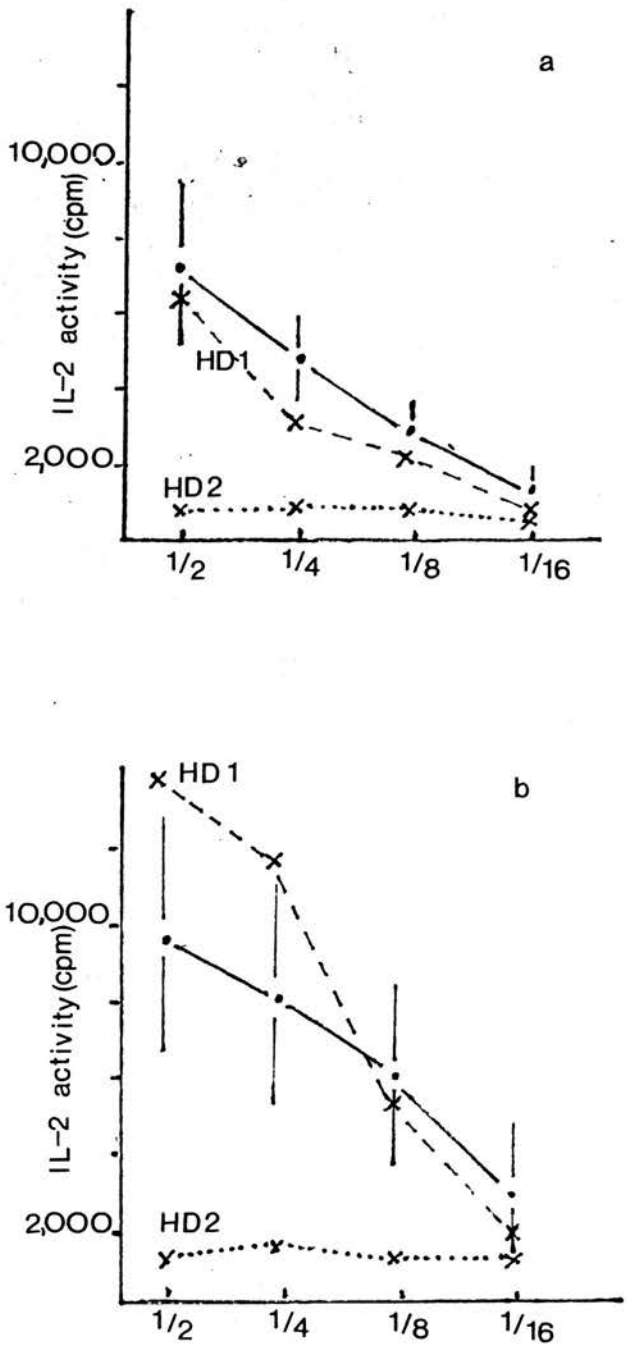

FIGURE 3.3 IL-2 ACTIVITY IN NORMAL AND HODGKIN'S DISEASE CELL CULTURE SUPERNATANTS

IL-2 activities in supernatants from 5 normal individuals (mean ± 1 S.D.) and two Hodgkin's disease patients are shown. In fig 3.3a IL-2 activities in supernatants from PHA stimulated cells are shown and in fig 3.3b IL-2 activities in supernatants from PHA stimulated cells cultured with indomethacin are shown. IL-2 activity was measured as described in methods, by culturing T-CFCs with culture supernatants at various concentrations. In this experiment control cultures gave the following responses: medium alone 540 c.p.m., PHA 0.5%v/v 834 c.p.m., T-CLL CM 50%v/v 12,508 c.p.m..

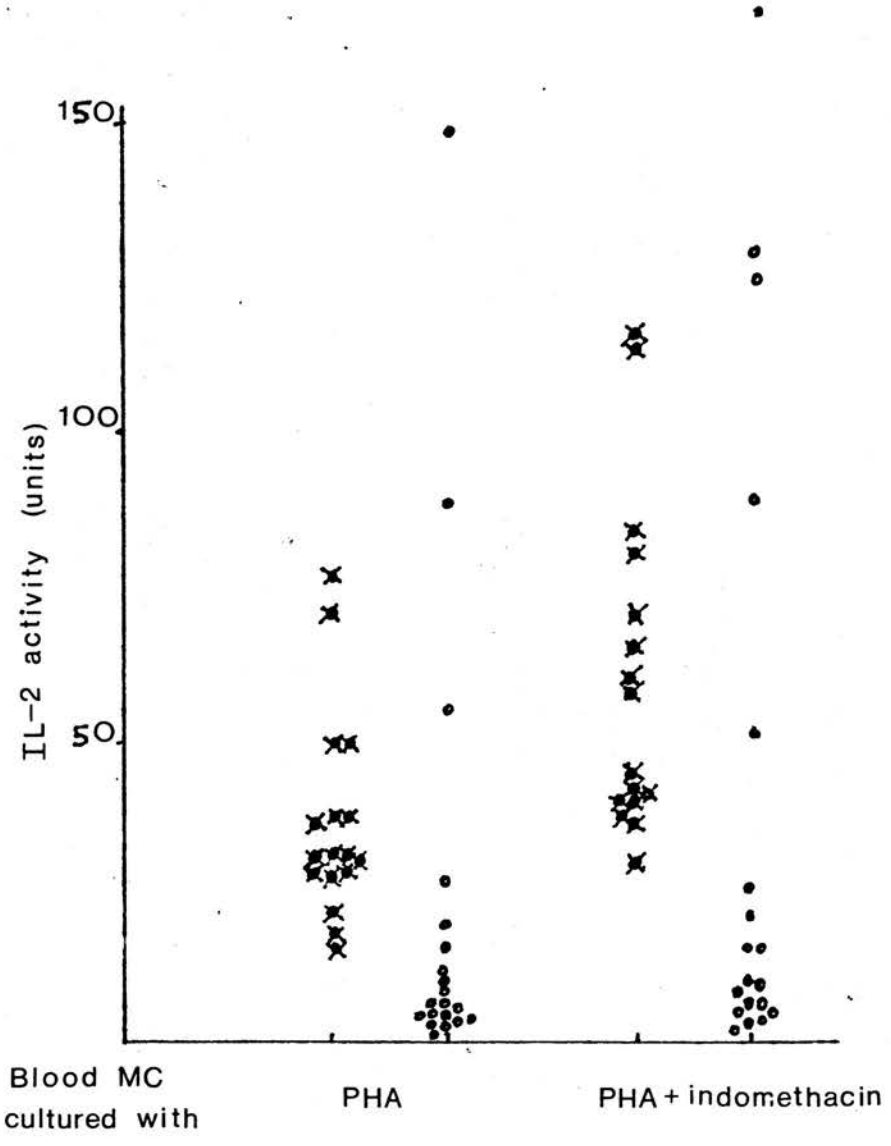

FIGURE 3.4 IL-2 ACTIVITY IN HODGKIN'S DISEASE CELL CULTURE SUPERNATANTS

Normal ✕ Hodgkin's Disease •

Colony formation in MC cultures supplemented with conditioned media:- The results presented above demonstrated a reduction in both colony formation and IL-2 production. Although no statistically significant correlation could be made between these two parameters the findings suggested that subnormal colony formation might in individual cases be consequent upon a defect in growth factor production. In order to further investigate this possibility colony formation was studied in cultures of HD patients MC supplemented with LyCM (as a source of IL-2) and MoCM (as a source of IL-1). Two cases have been studied (cases 18 and 28). Blood MC from patient 18 showed subnormal colony formation and low IL-2 production in response to PHA (with or without indomethacin). The addition of LyCM to cultures of this patient's MC increased colony formation to normal levels. MoCM also increased colony formation but to a lesser degree (table 3.10). Blood MC from patient 28 showed low IL-2 production following stimulation with PHA that was increased to normal by indomethacin. In this case colony formation was at the lower limit of the normal range in cultures containing PHA (with or without indomethacin) and was only moderately increased by the addition of LyCM or MoCM (table 3.10).

TABLE 3.10 THE EFFECTS OF CONDITIONED MEDIA ON COLONY FORMATION IN TWO PATIENTS WITH HODGKIN'S DISEASE

number of colonies/10^5cells

culture	PHA				PHA+indomethacin			
underlayer:	nil	LyCM	MoCM	Ly+MoCM	nil	LyCM	MoCM	Ly+MoCM
case 18	11	91	30	232	15	163	99	230
case 28	43	87	72	82	63	84	97	80
normals (4)	60-98	78-127	80-126	94-197	79-125	85-164	80-157	126-208

Blood MC were cultured at $1x10^5$cells/well. Figures are the mean values of triplicate cultures. Figures for normals give the range of values obtained from four normal blood samples cultured under identical conditions to the patient blood MC.

DISCUSSION

The reduction in colony formation in Hodgkin's disease shown in this study is consistent with previous reports (Douer and Sachs 1979, Schulhof et al 1981, Mukhopadhyaya et al 1983). A reduction in IL-2 production by blood MC has also been demonstrated for the first time. This reduction in IL-2 production, in Hodgkin's disease, has recently been confirmed in a study by Ford et al (1984). Reductions in colony formation and IL-2 production were present in newly diagnosed and treated patients at all disease stages and in all histological types.

The reduction in colony formation could not be attributed to a reduction in the percentage of T cells (%E rosette positive or %OKT3 positive cells) in blood mononuclear cell suspensions. Although there was wide variation in %T-cell counts, no correlation was found between %T cells and colony formation, with some patients with low T cells showing normal colony formation and some with normal T cells showing reduced numbers of colonies. In order to explain the reduction in colony formation it is therefore necessary to postulate either a specific reduction in the subset of T cells that have colony forming potential (i.e. T colony precursors) or a reduction in the ability of accesory cells to promote colony formation.

In previous studies it has been shown that colony

precursors include both T-helper (OKT4 or Leu3a positive) and T-cytotoxic/suppressor (OKT8 or Leu2 positive) cells (Claesson et al 1983), however whereas T-helper cells, which include the cells responsible for IL-2 production, may form colonies in response to PHA alone, T-suppressor/cytotoxic cells require IL-2 to be added to cultures. Thus it might be expected that colony formation in Hodgkin's disease would be related to changes in T-cell subsets and possibly through changes in IL-2 production. There was a positive correlation between colony formation and the percentage of T-helper (OKT4 positive) cells and a negative correlation between colony formation and the percentage of T-cytotoxic/suppressor (OKT8 positive) cells.

The observation that IL-2 production was reduced in Hodgkin's disease suggested that defective IL-2 production might be responsible for the subnormal colony formation. More direct evidence for this was obtained in an experiment in which blood MC, from a patient with markedly subnormal colony formation and IL-2 production, were shown to produce normal numbers of colonies when cultures were supplemented with IL-2 containing conditioned medium. In one other patient however IL-2 containing medium had only a small enhancing effect on colony formation. Further studies of colony formation in cultures supplemented with IL-2 are in progress in order to determine the extent to which lack of IL-2 production is responsible for the defect in colony formation in individual patients.

In patients with Hodgkin´s disease, no direct correlation could be made between colony formation and IL-2 production, or between IL-2 production and T-cell subsets. The lack of correlation between T-cell subsets and IL-2 production may be explained by the observation that the monoclonal antibodies OKT4 and OKT8 do not fully define functional T-cell subsets (OKT4 positive cells include cytotoxic and suppressor cells (Reinherz et al 1983), and OKT8 positive cells may include IL-2 producing cells (Luger et al 1982)). The lack of correlation between IL-2 production and colonies does however suggest that mechanisms other than reduced IL-2 production may be responsible for the failure of colony formation in some cases.

It has been suggested that defective colony formation is related to increased prostaglandin synthesis by blood MC in Hodgkin´s disease (Bockman 1980). In this study the prostaglandin inhibitor indomethacin was found to increase both colony formation and IL-2 production by most patients although even in the presence of indomethacin these both remained subnormal in the great majority of cases. Thus although prostaglandins may play a role, probably through reducing IL-2 production (Inouye et al 1980), it seems unlikely that this alone accounts for subnormal colony formation. Mukhopadhyaya et al (1983) have shown that blood MC culture supernatants from Hodgkin´s disease patients produce a factor that inhibits colony formation by normal blood MC. This factor has however not been characterised. Further studies are clearly needed to confirm that Hodgkin´s disease blood MC

produce colony inhibitory factors and to biochemically characterise these factors.

Conclusions

This study has confirmed previous observations of a reduction in T cell colony formation in Hodgkin´s disease. The mechanism of this reduction remains uncertain although evidence has been presented that suggests a reduction in IL-2 production is of major importance. It is possible that defects other than reduced IL-2 production, including alterations in the number of colony precursors and the production of inhibitory factors, may also contribute to subnormal colony formation in some patients. Further studies are in progress to determine the extent to which defects in colony formation can be corrected by the addition of IL-2 to cultures. If in vitro abnormalities of T-cell function, such as reduced colony formation, are a consequence of reduced IL-2 production and can be corrected by the addition of IL-2 then it is probable that impaired cellular immunity in vivo will also respond to IL-2 administration.

CHAPTER 4

INHIBITION OF T-LYMPHOCYTE COLONY FORMATION BY METHYLPREDNISOLONE

SUMMARY

Methylprednisolone was found to inhibit primary T cell colony formation at very low concentrations. This inhibition was shown to be a consequence of inhibition of growth factor production by accessory cells.

INTRODUCTION

Glucocorticoid hormones are known to inhibit lymphocyte functions both in vitro and in vivo. In some species, such as the mouse, glucocorticoids exert a direct lympholytic effect. In others , including man, lytic effects are not observed although steroids induce marked immunosuppression (Claman 1972, Cupps and Fauci 1982). In vitro studies of steroid action on human lymphocytes have shown that steroids can inhibit lectin induced lymphocyte transformation and proliferation (Nowell 1961, Smith et al 1977). These inhibitory effects have however been found to be highly dependent on culture conditions and to require concentrations of hormone greater than those usually obtained in vivo following glucocorticoid therapy (Webel and Ritts 1977, Kraft et al 1979 , Segel et al 1980).

Early studies of steroid action on lymphocyte mitogenesis attributed steroid action to a direct effect on lymphocytes through inhibition of metabolic functions, such as nutrient uptake, protein and nucleic acid synthesis, with consequent failure of the stimulated cells to enter or progress through the cell cycle (Mendelsohn et al 1977). More recently however it has been proposed that glucocorticoids inhibit lymphocyte proliferation not only through direct inhibitory effects on the metabolism of proliferating cells but also through inhibition of growth factor production by accesory cells (Gillis et al 1979a,b,, Larsson 1980, Palacios 1982).

The aim of the studies presented in this chapter was to determine whether T-cell colony formation was inhibited by the glucocorticoid, methylprednisolone, and to establish whether this effect was mediated by direct effects on colony forming cells or indirectly through inhibition of growth factor production by accesory cells.

METHODS

Blood mononuclear cells (MC) were isolated from heparinised venous blood by centrifugation over Ficoll-Hypaque as described in chapter 1.

Colony formation was carried out as described in chapter 1: For primary colony formation blood MC were suspended in RPMI 1640 containing 10% autologous plasma with agar added to 0.3%. Forty microlitres of this suspension (2 x 10^5cells) was plated onto an underlayer of 0.2ml of 0.5% agar in 1640 with 10% autologous plasma and 1% v/v PHA. All cultures were in 17mm diameter wells in multiwell dishes.

For secondary colony formation 2 x 10^5colony cells, harvested from 6-7 day primary cultures, were plated onto underlayers containing 50% conditioned medium (see below).

All cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO2 in air.

Methylprednisolone sodium succinate (MPS) (Upjohn) was added to culture underlayers to give final concentrations between $10^{-2}$and 10^{-10}M.

Colonies, defined as aggregates of more than 40 cells, were counted after incubation for 6 days. In some experiments both clusters (aggregates of 4-40 cells) and colonies were counted and the number of cells in each cluster and colony were determined by examination of 50-100 cell aggregates at x 100 magnification.

Conditioned Medium (CM): CM was prepared by incubating 2×10^6 freshly isolated MC in 2ml of RPMI 1640 containing 10% autologous plasma and 1%v/v PHA for 48 hours. Culture supernatants were harvested following centrifugation at 100g for 20 minutes and stored at $-20^{o}C$. This conditioned medium is referred to as PHA-CM. CM was also prepared as described above but with the addition of MPS to cultures, at various concentrations, throughout the 48 hour incubation period. This CM is referred to as MPS/PHA-CM. In some experiments conditioned medium was prepared as described above but with Monocyte CM (MoCM, 15% v/v) added to cultures of MC in addition to PHA.

Assays for interleukin 2 (IL-2) activity in CM were carried out as described in chapter 2: Colony cells harvested from primary cultures were suspended at 2×10^5 cells/ml in RPMI 1640 with 20% heat inactivated human serum and 0.2ml of this suspension was aliquoted into a 17mm diameter flat bottomed well. A further 0.2ml of either RPMI 1640 (with or without 1% v/v PHA) or CM was added to the well. Cultures were incubated for 4 days and then pulsed for 4 hours with 0.4 microcurie of 3H-thymidine. Cells were then harvested onto 25mm Whatman GF/C filters, washed twice with phosphate buffered saline followed by one wash with 5% TCA and two washes with absolute ethanol. The filters were then placed in scintillant for measurement of acid-precipitable 3H-thymidine incorporation.

1) INHIBITION OF PRIMARY COLONY FORMATION BY MPS

MPS inhibited primary colony formation, in a dose dependent manner, at concentrations between 10^{-10}M and 10^{-2}M (table 4.1). Inhibition of colony formation was maximal when MPS was added at the beginning of culture; delay of as little as one hour in the addition of MPS resulted in substantially diminished inhibition (table 4.2).

TABLE 4.1 INHIBITION OF PRIMARY T LYMPHOCYTE COLONY FORMATION BY MPS

Concentration of MPS [M]	Colony number (mean±1S.D.) expt.1	expt.2	expt.3	expt.4	expt.5
0	188±26	124±19	116±15	165±36	155±9
10^{-10}	84±31	71±7	not tested		
10^{-8}	81±12	49±6	80±12	88±18	40±2
10^{-6}	42±11	29±12	48±17	21±8	3±3
10^{-4}	3±2	19±12	11±3	3±2	0
10^{-2}	0	6±3	0	0	0

Figures are the mean value ± 1 S.D. of colony counts from quadruplicate cultures from 5 different donors.

TABLE 4.2 THE EFFECT OF THE TIME OF ADDITION OF MPS TO CULTURES ON PRIMARY COLONY FORMATION

Incubation conditions	Number of colonies (mean ± 1 S.D.) expt.1	expt.2	expt.3	expt.4
No MPS	115±23	73±11	115±5	110±13
MPS added after				
0 hours	0	6±4	0	3±5
1 hour	124±30	26±8	45±11	39±12
24 hours	113±17	58±6	51±4	73±13
48 hours	not tested		52±13	67±11

Figures are the mean values ± 1 S.D. of quadruplicate cultures from 4 different donors. MPS (10 microlitres 10^{-5}M) were added by pipetting onto the culture overlayers, either at the beginning of culture (0 hours), or after various periods of incubation.

Evidence that inhibition was the result of specific glucocorticoid interaction with high affinity cellular receptors was given by experiments in which cultures were incubated with the competetive glucocorticoid antagonist cortexolone. Cortexolone binds specifically to cytoplasmic glucocorticoid receptor blocking binding by MPS but has little pharmacological activity itself. The addition of cortexolone alone had only a small inhibitory effect, but when present in cultures containing MPS substantially reduced the inhibitory effects of MPS on colony formation (table 4.3).

TABLE 4.3 THE EFFECT OF CORTEXOLONE ON COLONY FORMATION IN MPS TREATED CULTURES.

Culture conditions	Number of colonies (mean ± 1S.D.)	
	expt.1	expt.2
No MPS	127±7	68±13
MPS 10^{-6}M	25±15	0
Cortexolone 10^{-4}M	95±13	35±15
MPS 10^{-6}M + Cortexolone 10^{-4}M	77±2	34±15

Figures are the mean colony counts ± 1 S.D. of quadruplicate cultures from 2 different donors.

In primary cultures MPS reduced not only the number of colonies but also reduced colony size. When cultures were scored counting both the number of colonies (cell aggregates >40cells) and clusters (cell aggregates 4-40 cells), there was found to be no change in the total number of aggregates developing but a marked diminution in mean aggregate size. Thus in the absence of MPS most aggregates that developed were of greater than 40 cell size and scored as colonies whereas in the the presence of MPS the majority of aggregates were less than than 40 cells in size and were scored as clusters (plates 4.1,4.2, tables 4.4,4.5).

TABLE 4.4 THE EFFECT OF MPS ON THE TOTAL NUMBER OF COLONIES AND CLUSTERS AND COLONY SIZE.

Culture conditions	colonies	clusters	total colonies + clusters	no. cells/ aggregate
No MPS	116±20	40±4	156±22	190±29
10^{-8}M MPS	47±9	86±12	131±20	86±5
10^{-6}M MPS	28±9	125±31	151±38	50±9

Figures are the mean values ± 1 S.E. of 4 experiments using MC from different donors. In each experiment triplicate or quadruplicate cultures were examined and colonies (>40 cells) and clusters (4-40 cells) were counted. Colony and cluster size (number of cells/aggregate) was determined by examining between 40 and 100 colonies and clusters, in steroid treated and untreated cultures, in each experiment, at x 100 magnification.

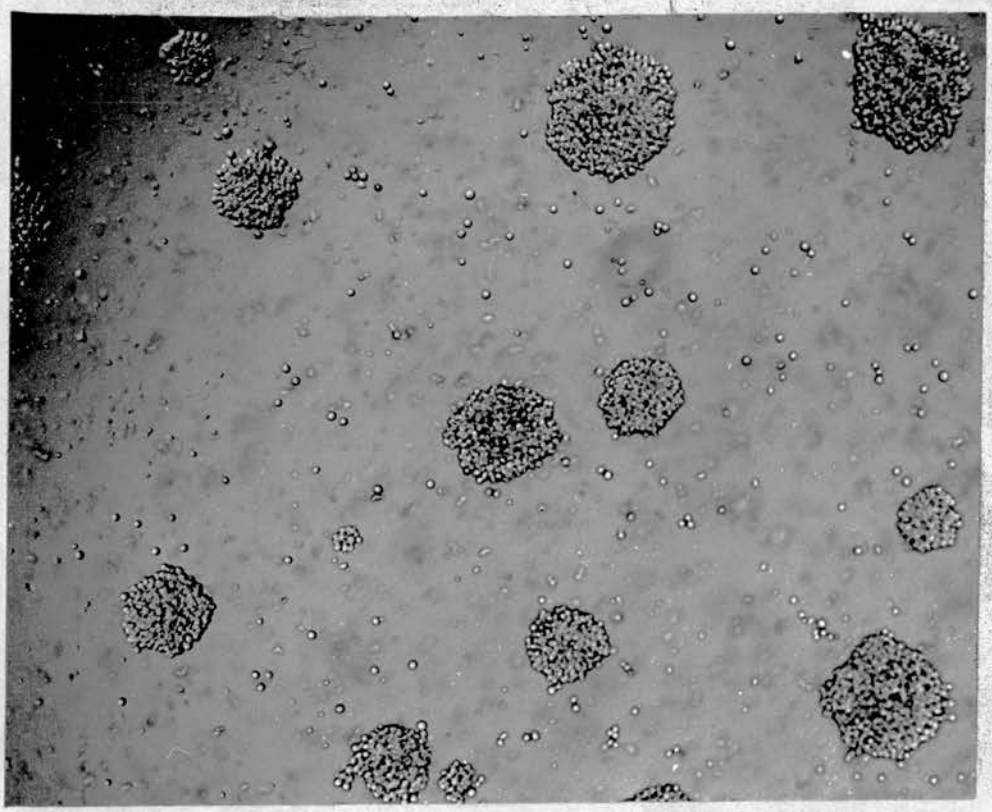

PLATE 4.1 T-LYMPHOCYTE COLONIES IN PRIMARY CULTURE
Colonies grown in the absence of MPS (original x100)

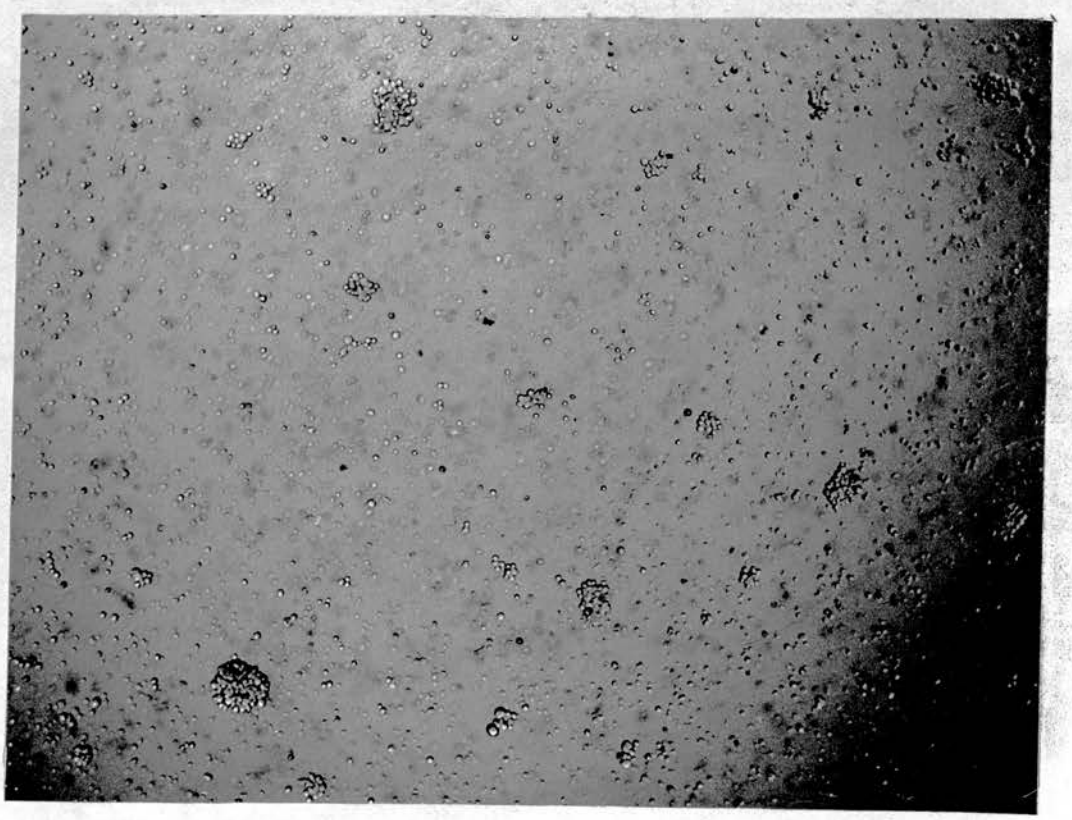

PLATE 4.2 T-LYMPHOCYTE COLONIES IN PRIMARY CULTURE
Colonies grown in the presence of 10^{-6}M MPS (original x100)

TABLE 4.5 THE EFFECT OF MONOCYTE CONDITIONED MEDIUM (MoCM) AND LYMPHOCYTE CONDITIONED MEDIUM (PHA-CM) ON MPS TREATED PRIMARY COLONY FORMATION

	Colony number (mean ± 1S.D.)		
Culture conditions:	no additive	PHA-CM	MoCM
expt.1			
no MPS	209±19	220±16	195±13
10^{-8}M MPS	121±11	231±25	147±15
expt.2			
no MPS	122±21	142±16	138±7
10^{-8}M MPS	89±5	135±6	112±4
expt.3			
no MPS	232±26	232±11	225±14
10^{-8}M MPS	89±7	243±18	208±6

Figures are mean colony counts ± 1 S.D. of triplicate cultures using blood MC from 3 different donors. PHA-CM was included in culture underlayers at 50% v/v and MoCM at 15% v/v.

In order to determine whether the reduction in the size of colonies was due to a direct effect of the hormone on colony forming cells or secondary to inhibition of growth factor production by accesory cells, experiments were performed in which colony formation was measured with conditioned medium (CM) added to cultures . Both MoCM

(containing IL1) and PHA-CM(containing IL2) were found to reduce MPS induced inhibition of colony formation. The addition of PHA-CMcompletely reversed the inhibition due to MPS; MoCM partially reversed inhibition (table 4.5). In a further experiment, in which both colony and cluster formation and colony size were measured, the addition of PHA-CMto MPS treated cultures was found to restore colony formation to normal and to prevent the reduction in colony and cluster size (table 4.6).

TABLE 4.6 THE EFFECT OF PHA-CM ON COLONY NUMBER AND SIZE IN MPS TREATED PRIMARY CULTURES

		number of cells/colony-cluster (% of total counted)			Number of
	Colony count	5-20	21-40	>40	aggregates examined
No MPS	232±16	15	23	62	56
10^{-8}M MPs	83±7	34	38	28	64
PHA-CM	232±11	20	27	53	84
PHA-CM+MPS	243±18	25	30	45	92

Colony counts are the mean value ± 1 S.D. of triplicate cultures. Colonies were scored by examining three fields per well at x40 magnification. Colony and cluster (aggregate) size was evaluated by examining cultures at x100 magnification; the figures for number of aggregates examined are the number of colonies and clusters seen in three high power (x100) fields.

2) INHIBITION OF SECONDARY COLONY FORMATION BY MPS

Further evidence that MPS mediated inhibition of colony formation was due to inhibition of growth factor production was provided by studies of secondary colony formation. In order to determine whether proliferation of colony cells in secondary culture was inhibited by MPS, experiments were performed in which T-CFCs were cultured with conditioned medium (PHA-CM) prepared by PHA stimulation of autologous freshly isolated blood MC . Parallel cultures were also carried out using CM from the same autologous MC stimulated with PHA in the presence of MPS (MPS/PHA-CM).

Secondary colony formation occured only in cultures containing conditioned medium. In the presence of PHA-CM, MPS was inhibitory but only at high concentrations of MPS ($>10^{-8}$M); the inhibitory effect even at these high concentrations was much less marked than the inhibition found in primary cultures using the same concentration of MPS. However when cultures were performed using CM prepared from MPS treated cells (MPS/PHA-CM) colony formation was reduced even when blood MC used to prepare CM had been treated with as little as 10^{-10}M MPS (table4.7). In these secondary cultures the addition of MPS, even when PHA-CM was present, did however reduce colony size (table 4.8). This finding suggested that MPS may exert a direct inhibitory effect on colony cells in addition to inhibiting the production of growth factors.

TABLE 4.7 INHIBITION OF SECONDARY T-LYMPHOCYTE COLONY FORMATION BY MPS.

	number of colonies (mean ± 1 S.D)			
	expt.1	expt.2	expt.3	expt.4
Culture conditions				
PHA-CM, No MPS	113±32	84±8	187±24	115±7
PHA-CM + 10^{-10} M MPS	not done	97±8	201±23	121±15
+ 10^{-8} M MPS	83±22	87±9	181±11	114±23
+ 10^{-6} M MPS	74±9	79±11	120±16	81±12
+ 10^{-4} M MPS	not done	68±9	81±15	58±11
MPS/PHA-CM				
10^{-10}M	not done	91±15	142±17	103±10
10^{-8} M	60±15	21±9	72±16	69±20
10^{-6} M	16±11	2±1	23±9	47±6
10^{-4} M	5±3	0	0	9±6

Figures are the mean colony counts ± 1 S.D. of triplicate or quadruplicate cultures in each experiment. CM were included in culture underlayers at 50% v/v concentration. PHA-CM was the culture supernatant from PHA stimulated blood MC. MPS/PHA-CM was the culture supernatant of blood MC stimulated with PHA in the presence of various concentrations of MPS.

TABLE 4.8 THE EFFECT OF MPS ON COLONY SIZE IN SECONDARY CULTURE.

	Number of colonies	Number of clusters	Size of aggregates
Culture conditions			
PHA-CM, No MPS	104±9	32±5	79
PHA-CM + 10^{-8}M MPS	102±19	41±11	44
PHA-CM + 10^{-6}M MPS	83±10	49±15	35

Figures are the mean colony (aggregates >40cells) and cluster (aggregates 4-40 cells) counts ± 1 S.D. of triplicate cultures. The mean size of cell aggregates was measured by direct examination of cultures at x100 magnification and counting the number of cells in 50- 100 colonies and clusters.

When colony cells were incubated in secondary culture in suspension and proliferation measured by thymidine incorporation a vigorous proliferative response was seen with PHA-CM, however MPS/PHA-CM showed no T-cell stimulatory (IL-2) activity (table 4.9). In suspension culture the addition of MPS to cultures containing PHA-CM had no measurable inhibitory effect on proliferation measured by thymidine incorporation (table 4.9). In these experiments the IL-2 activity of culture supernatants from blood MC stimulated with PHA and MoCM, with or without MPS, was also measured. This was done in order to determine whether the inhibitory effects of MPS on T cell growth factor production could be blocked by adding exogenous IL-1 (present in MoCM). The results of these experiments showed that MPS substantially reduced IL-2 production even when MoCM was added to cultures. In cultures from MPS treated, PHA and MoCM stimulated, cells detectable amounts of IL-2 were however still measurable (table 4.9) . This finding demonstrates that while MPS probably directly inhibits the IL-2 producing cell, it is also possible that indirect effects through inhibition of monocyte IL-1 production may also be involved .

TABLE 4.8 INHIBITION OF IL-2 PRODUCTION BY MPS.

Source of CM	Additive to assay system	IL-2 activity (c.p.m.)
MC + PHA (PHA-CM)	nil	8,769±635
	MPS	7,965±422
MC + PHA + MPS (MPS/PHA-CM)	nil	885±122
MC + PHA + MoCM	nil	12,650±1076
	MPS	10,894±756
MC + PHA + MOCM + MPS	nil	3,065±593

Figures are the mean values ± 1 S.D. of triplicate cultures in a single experiment. These findings were confirmed in two further identical experiments. Responses of T cells in control cultures were : medium alone (RPMI 1640 no CM) 1068±322 c.p.m., PHA 0.5% 1465±345 c.p.m., MoCM 15% v/v 965+256, PHA + MoCM 2450±240.

DISCUSSION

These studies demonstrate that MPS inhibits T lymphocyte colony formation at very low concentrations (10^{-10} M-10^{-8} M) that correspond to concentrations of endogenous glucocorticoid such as cortisol of around 2×10^{-8} to 2×10^{-6} M (Claman 1972). These concentrations overlap the physiological range and fall well within the range required to saturate the high affinity cytoplasmic glucocorticoid receptors (Lippman et al 1973, Smith et al 1977). The observation that inhibitory effects were blocked by cortexolone, which competes with active glucocorticoids for these receptors, confirms that inhibition is due to specific binding to cytoplasmic receptors. In a recent study, using hydrocortisone, Claesson and Ropke (1983) have confirmed the sensitivity of T colony formation to extremely low concentrations of glucocorticoids.

In primary cultures conditioned medium from PHA stimulated MC (PHA-CM) almost completely abrogated the inhibitory effects of MPS. This finding suggested that inhibitory effects of MPS were largely due to inhibition of growth factor production by accessory cells. This possibility was confirmed by studies of secondary colony formation, which is dependent upon the addition of exogenous growth factor (most probably IL-2) to cultures. In secondary culture it was found that colony formation in cultures containing PHA-CM showed little or no inhibition in the presence of MPS.

However, cultures supplemented with conditioned medium from MPS treated, PHA stimulated MC (MPS/PHA-CM) showed either reduced or no colony formation. Measurement of IL-2 activity in culture supernatants confirmed a marked reduction or absence of IL-2 activity in MPS/PHA-CM. Similar findings have been demonstrated by Gillis et al (1979a,b) who demonstrated that dexamethasone inhibits lymphocyte proliferation by reducing IL- 2 activity in culture supernatants of lectin stimulated mouse and human lymphocytes.

MoCM (containing IL-1 activity) was less effective than PHA-CM (containing IL-2) in preventing inhibition of primary colony formation and was found to only slightly increase IL-2 activity in culture supernatants from MPS treated PHA stimulated MC. These findings, together with the observations discussed in the preceding paragraph, suggested that the major inhibitory action of MPS in colony formation was to inhibit IL-2 production (Krajewski and Wyllie 1981). However it has recently been shown that inhibition of IL-2 production by steroids is in part a consequence of inhibition of IL-1 production by monocytes (Smith 1980, Snyder and Unanue 1982), as well as a consequence of direct inhibition of IL-2 synthesis by IL-2 producing T-helper cells (Palacios and Sugawara 1982, Palacios 1982). It is therefore probable that both IL-1 and IL-2 production are targets for inhibition by glucocorticoids.

Experiments in which MPS was added to cultures at varying

times showed that early addition of MPS was necessary to inhibit colony formation. These findings are similar to those of Webel and Ritts (1977) and Robertson et al (1981) who found that early addition, or preincubation of cultures with MPS was necessary to inhibit thymidine incorporation into lectin stimulated cells. This is consistent with the hypothesis that MPS acts mainly through inhibition of growth factor production since it is known that both monocyte derived colony stimulatory factors, such as IL-1 (Claesson et al 1977b, Rosenszajn et al 1981), and lymphocyte derived factors, such as IL-2 (Gillis et al 1978b), are produced predominantly in the early stages of culture.

The possibility that MPS may directly inhibit proliferation of growth factor responsive colony cells has not been completely excluded in these studies. It is however unlikely that this is a major effect since the inhibitory effects of MPS were largely blocked by the addition of PHA-CM (containing IL- 2) to cultures and it has been reported by others that glucocorticoids have no effect on the ability of lectin activated T cells to respond to IL-2 (Gillis et al 1979a,b, Larsson 1980, Palacios 1982). Claesson and Ropke (1983) have however shown that the proliferation of a subpopulation of colony cells (Leu2 positive T cells) may be directly inhibited by hydrocortisone even in the presence of IL-2. This observation may explain the reduction in size of colonies I have observed in secondary cultures containing MPS (table 4.8).

The question arises as to the relevance of these in vitro findings on the effect of glucocorticoids on proliferating T cells in vivo. The lymphopenia which follows systemic administration of steroid in man is more readily attributable to sequestration of circulating lymphocytes than to any effect on their proliferation (Fauci and Dale 1974, Cupps and Fauci 1982). However the proliferation of T cells to form colonies in agar, observed here, may be considered analogous to the proliferation of T cells in lymphoid tissue and inflammatory foci in vivo where proliferation occurs in a cell rich milieu that includes accessory cells such as macrophages. In such circumstances it is propsed that the major site of action of glucocorticoid hormones is to inhibit production of soluble growth factors by accessory cells rather than due to direct effects on the proliferating T cells themselves.

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PUBLICATIONS

The following work connected with this thesis has been published

Krajewski A.S. and Wyllie A.H. (1981) Inhibition of human T lymphocyte colony formation by methylprednisolone Clin.exp.Immunol. 46, 206-213

Colledge Nicola R., Krajewski A.S., Smyth J.F. and Wyllie A.H. (1982) Action of deoxycoformycin on human T cell colonies in vitro
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Inhibition of human T lymphocyte colony formation by methylprednisolone

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(Accepted for publication 17 April 1981)

SUMMARY

Methylprednisolone, at very low concentrations, inhibits colony formation by PHA-stimulated human blood lymphocytes *in vitro*. The inhibition is exerted at an early stage in transformation. It results not from direct effects on colony-forming cells but from inhibition of production of a soluble factor produced by accessory cells.

INTRODUCTION

Glucocorticoid hormones are well known to inhibit the function of lymphoid cells *in vivo* (see review by Claman, 1972), but attempts to analyse their mode of action *in vitro* have generated conflicting results. Direct lethal effects of low concentrations of steroid on lymphocytes *in vitro* are observed only in some species, yet the resistant species, which include man, show prominent immunosuppression on treatment with steroid. Lymphocyte transformation and proliferation, induced *in vitro* by exposure to lectins, has also been investigated as a potential target for steroid action. Whilst some groups have demonstrated inhibition of transformation (Nowell, 1961; Smith *et al.*, 1977; Robertson *et al.*, 1981), others have found little effect (Kraft, Thomson & Atkins, 1979; Segel *et al.*, 1980).

In order to investigate further the effect of glucocorticoids on lymphocyte transformation we have studied the effect of methylprednisolone (MPS) on phytohaemagglutinin (PHA) induced human T lymphocyte colony formation in agar gels (Rozenszajn, Kalechman & Shoham, 1975; Riou *et al.*, 1976). Colony formation was chosen as an assay for proliferation in preference to conventional techniques for measuring lymphocyte responses, such as tritiated thymidine incorporation or blast transformation in liquid culture, as there is evidence that colony-forming assays may more accurately reflect inhibitory drug effects *in vivo* than other *in vitro* techniques (Roper & Drewinko, 1976).

In this paper we show that colony formation by human peripheral blood lymphocytes is sensitive to low concentrations of MPS. Moreover, colony formation is shown to provide an assay system capable of dissecting the point of action of glucocorticoids with considerable precision, despite the use of culture conditions which were incompletely defined chemically.

MATERIALS AND METHODS

Mononuclear cells. Mononuclear cells were isolated from heparinized venous blood from healthy volunteers by centrifugation over Ficoll–Hypaque (Böyum, 1968). Freshly isolated mononuclear cells were washed twice in RPMI 1640.

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0009-9104/81/1000-0206$02.00

One-step culture procedure (modified from Goube de la Forest *et al.*, 1979). Mononuclear cells were suspended in RPMI 1640 supplemented with 10% autologous plasma ('growth medium') with agar added to 0·3%. Forty microlitres of this suspension (2×10^5 cells) were plated onto an underlayer of 0·2 ml of 0·5% agar in growth medium and 20 μg phytohaemagglutinin M (PHA) (DIFCO). All cultures were in 17-mm-diameter wells in Multiwell dishes (Linbro 76-033-05).

Two-step culture procedure (modified from Rozenszajn *et al.*, 1975). Five million mononuclear cells were incubated for 18 hr in 5 ml RPMI 1640, 10% autologous plasma and 500 μg PHA. The cells were then harvested by centrifugation, washed three times, disaggregated by repeated passage through a fine Pasteur pipette, and resuspended in 0·3% agar in growth medium. One millilitre of this suspension (5×10^5 cells) was plated on to an underlayer of 1 ml of 0·5% agar in growth medium and 100 μg PHA. Cultures were carried out in 35-mm plastic Falcon petri dishes.

Secondary cultures (Goube de la Forest *et al.*, 1979). Cells growing in colonies in primary (one-step) cultures were harvested after 7 days, washed three times in RPMI 1640, disaggregated by repeated passage through a fine Pasteur pipette and resuspended in 0·3% agar in growth medium. Forty microlitres (2×10^5 cells) were pipetted on to a 0·2 ml underlayer of 0·5% agar growth medium with 20 μg PHA. For secondary cultures underlayers contained either 50% PHA-conditioned medium (CM) or 2×10^5 freshly isolated allogeneic mononuclear cells.

All cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air.

Methylprednisolone sodium succinate (MPS) (Upjohn) was added in RPMI 1640 to underlayers to give a final concentration of between 10^{-10} and 10^{-4} M.

Colonies, defined as aggregates of more than 40 cells, were scored after incubation for 6 days. In some experiments both clusters (4–40 cells) and colonies (more than 40 cells) were scored. Colony and cluster size was determined by examination at × 100 magnification.

Conditioned medium (CM). CM was prepared by incubating 2×10^6 freshly isolated mononuclear cells in 2 ml growth medium and 200 μg PHA for 48 hr. Following centrifugation at 100 *g* for 20 min, culture supernatants were harvested, sterilized by millipore filtration and stored at −20°C for up to 2 weeks before use. In some experiments conditioned medium was prepared as above but from cultures to which MPS had been added at various concentrations throughout the 48-hr incubation period (MPS-CM).

Identification of lymphocyte subclasses. T and B lymphocytes were identified by rosetting with sheep red blood cells and immunofluorescence respectively. Monocytes were identified by phagocytosis and staining with non-specific esterase and acid phosphatase (Dewar, Krajewski & Murray, 1980; Krajewski & Dewar, 1981).

^{3}H-thymidine uptake by T cell blasts. Cells harvested from primary agar cultures (T cell blasts) were suspended at 10^5 cells/ml in RPMI 1640 with 20% human serum and 0·2 ml of this suspension was aliquoted into flat-bottomed wells in multiwell plates (Linbro 76-033-05). A further 0·2 ml of either RPMI 1640 containing 1% PHA or conditioned medium was added to individual wells. Cultures were incubated for 4 days and then pulsed for 4 hr with 1 μCi of ^{3}H-thymidine ([methyl-^{3}H]thymidine, 40–60 Ci/mmol, Radiochemical Centre, Amersham). Cells were collected on 25-mm Whatman GF/C filters, washed twice with phosphate-buffered saline followed by one wash with 5% TCA and two washes with absolute alcohol. The filters were then placed in scintillant for measurement of acid-precipitable ^{3}H-thymidine incorporation (c.p.m.)

RESULTS

Inhibition of primary colony formation by MPS

In one-step cultures, MPS was found to inhibit colony formation at concentrations ranging from 10^{-10} to 10^{-4} M (Fig. 1). Maximum inhibition was obtained only if MPS was present from the onset of PHA stimulation; delay of only 1 hr resulted in substantially less inhibition (Table 1).

In two-step cultures, MPS at the same concentrations inhibited colony formation only if the cells were incubated with MPS during the first step before plating out in agar; incorporation of MPS into agar after preincubation without MPS did not reduce colony formation. Similarly, addition of MPS to agar did not affect further the number of colonies obtained from cells treated in the preceding 48 hr with MPS.

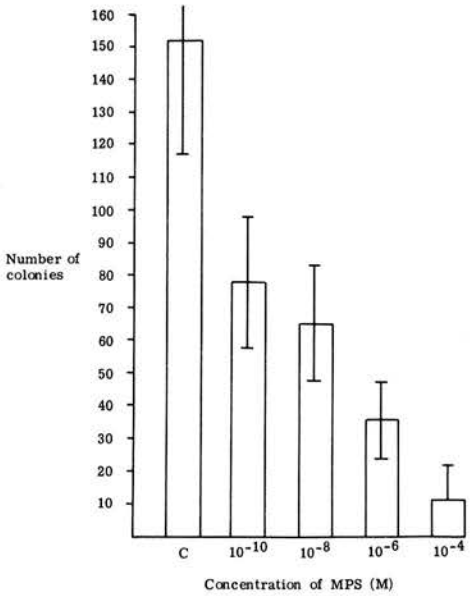

Fig. 1. Inhibition of colony formation by MPS in one-step cultures. Mean ± s.d. of eight lymphocyte cultures in each experiment.

Evidence that inhibition of colony formation was the result of specific glucocorticoid interaction with high-affinity cellular receptors was given by experiments in which cultures were incubated with 10^{-6} M MPS together with the glucocorticoid antagonist cortexolone at 10^{-4} M. By themselves these high concentrations of cortexolone led to a small reduction in the number of colonies, but the inhibitory effects of MPS were substantially reversed.

Table 1. Effect of time of addition of MPS to cultures on colony formation

	Number of colonies ± s.d.			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
No MPS added	115 ± 23	73 ± 11	115 ± 5	110 ± 13
Time MPS added*				
0 hr	0	6 ± 4	0	3 ± 5
1 hr	124 ± 30	26 ± 8	45 ± 11	39 ± 12
24 hr	113 ± 17	58 ± 6	51 ± 4	73 ± 13
48 hr	n.d.	n.d.	52 ± 13	67 ± 11

* Ten microlitres of 10^{-5} M MPS were added to cultures either at the beginning of culture (0 hr) or after varying periods of incubation, by pipetting MPS onto the cell layer. In each experiment results are the mean ± s.d. of four cultures.

n.d. = Not done.

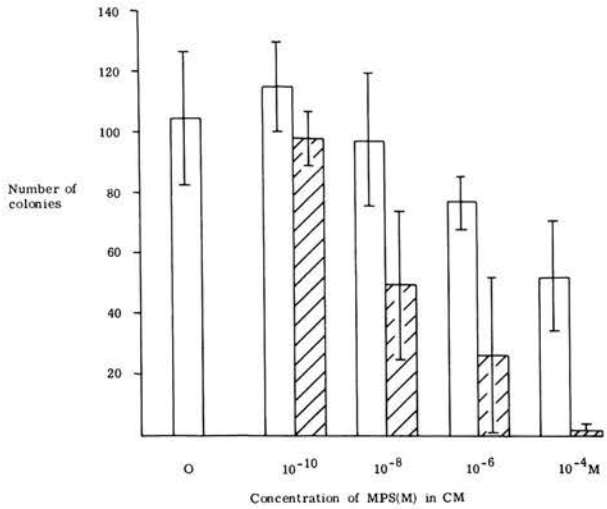

Fig. 2. Effect of MPS on secondary cultures. (□) Culture with active CM and freshly added MPS, (▨) culture with CM made in the presence of MPS (MPS-CM). Mean ± s.d. of 10 lymphocyte cultures in each experiment.

Effect of MPS on secondary colony formation

Cells harvested from primary cultures constituted a relatively homogeneous population of large cells, with prominent nucleoli, frequent mitotic figures and T cell surface characteristics. Thus in these experiments over 70% of cells formed rosettes with sheep red blood cells; no phagocytic cells or cells with surface immunoglobulin were detectable; and the cells were esterase- and acid phosphatase-negative. Accordingly, we refer to them as T cell blasts.

Such T cell blasts, harvested and reseeded into agar proved incapable of producing secondary colonies without addition of other factors. This has been shown to be the result of depletion of accessory cells originally present in the primary cultures (Goube de la Forest *et al.*, 1979).

Table 2. Effect of CM on proliferation of T cell blasts* in agar and liquid culture

Agar culture†	Colonies (± s.d.)
Underlay with:	
PHA	0
CM	292 ± 24
CM + 10^{-8} M MPS	331 ± 60
Liquid culture‡	**c.p.m. × 10^3 (± s.d.)**
Underlay with:	
PHA	0·4 ± 0·1
CM	63·0 ± 6·4
CM + 10^{-8} M MPS	58·9 ± 2·8

* T cell blasts were harvested from primary cultures.

† Standard secondary cultures. Results are mean ± s.d. of three cultures.

‡ ^{3}H-thymidine uptake was assessed as described under Materials and Methods. Results are mean ± s.d. of three cultures.

Proliferation of reseeded T cell blasts was observed, however, when either allogeneic freshly isolated blood mononuclear cells or conditioned medium (CM) from blood mononuclear cell cultures were incorporated in culture underlayers. This system thus allowed us to separate direct effects of MPS on proliferation of T cell blasts from indirect effects on accessory cells responsible for the production of colony-stimulating factors.

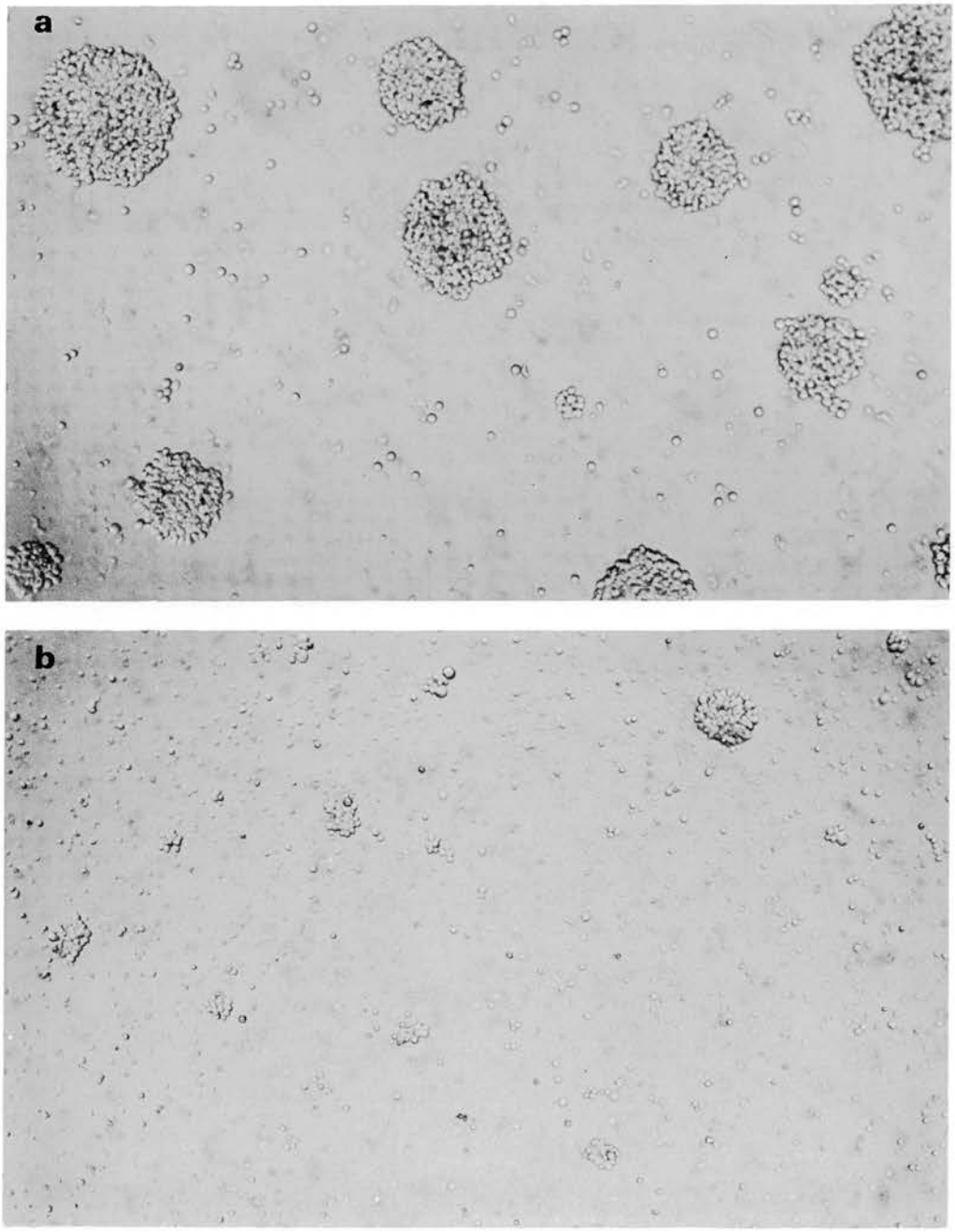

Fig. 3. (a) Human T lymphocyte colonies in primary culture, grown in the absence of MPS. (Original × 100.) (b) Human T lymphocyte colonies in primary culture, grown in the presence of 10^{-6} M MPS. (Original × 100.)

It was found that MPS added to secondary cultures containing active CM had little effect on colony formation, except at high concentrations, whereas conditioned medium prepared from MPS-treated cells (MPS-CM) showed markedly decreased ability to support secondary colony formation. The activity of conditioned medium was decreased by about 50% by culturing mononuclear cells used in its preparation with 10^{-8} M MPS, whereas secondary colony formation, in the presence of active CM, was not inhibited by the addition of the same concentration of MPS to culture underlayers (Fig. 2, Table 2).

Similarly, ^{3}H-thymidine incorporation by T cell blasts harvested from primary culture and incubated in suspension into CM was not inhibited (Table 2).

In an analogous manner, experiments using allogeneic mononuclear cell feeder layers showed that MPS incorporated into the culture underlayers inhibited *in situ* production of colony-stimulating activity at concentrations as low as 10^{-10} M.

Effect of MPS on T cell colony size

In primary cultures MPS consistently reduced the numbers of colonies. This effect appeared to be mainly the result of a reduction in the size of cell aggregates rather than an overall reduction in the number of colony-forming cells initially stimulated to proliferation; i.e. in the presence of MPS, clonogenic cells showed a reduced rate of proliferation resulting in cell aggregates of less than 40 cells (clusters) developing, whereas in the absence of MPS the majority of cell aggregates contained more than 40 cells (colonies) (Fig. 3a, b).

In order to determine whether this reduction in the size of cell aggregates was the result of direct inhibition of clonogenic cell proliferation or inhibition of growth factor production by accessory cells within the primary culture, CM was incorporated into culture underlayers in primary cultures. The results of a typical experiment are shown in Table 3. In this experiment, 10^{-8} M MPS substantially reduced the number of colonies (aggregates >40 cells). In the presence of CM no reduction in the number of colonies was seen (Table 3a). *In the same experiment* the total number of clusters and colonies developing in the presence of MPS and in control cultures was also determined (Table 3b). In the presence of MPS the total number of cell aggregates was similar to control cultures; however, in MPS-treated cultures only 28% of aggregates were of colony size (>40 cells)

Table 3. Effect of MPS on colony and cluster size in primary culture

	No. of colonies*			
(a)				
No MPS	232±26			
10^{-8} M MPS	89±7			
CM	232±11			
CM+10^{-8} M MPS	243±18			
	No. of cells/colony–cluster (% of total counted)			
	5–20	21–40	>40	Total no. counted†
(b)				
No MPS	15	23	62	56
10^{-8} M MPS	34	38	28	64
CM	20	27	53	84
CM+10^{-8} M MPS	25	30	45	92

* Figures are mean ± s.d. of three cultures.

† The total number of colonies and clusters and their size was evaluated by examining cultures at ×100 magnification. Figures are numbers seen in three fields.

whereas in control cultures 62% of aggregates were of colony size. The addition of CM to cultures increased the total number of cell aggregates developing in both control and MPS-treated cultures and increased the number of colony-size aggregates to near control levels in MPS-treated cultures (Table 3b).

These experiments demonstrate, as expected from previous data, that inhibition of primary colony formation by MPS is reversible by CM and probably results from inhibition of accessory cell function rather than direct effects on colony-forming cells.

DISCUSSION

The results reported here show that MPS inhibits human T lymphocyte colony formation *in vitro*. This inhibition is demonstrable at extremely low concentrations (10^{-10}–10^{-8} M), corresponding to concentrations of endogenous glucocorticoid such as cortisol of around 2×10^{-8} to 2×10^{-6} M (Claman, 1972) which overlap with the physiological range. Since the growth-supporting medium included autologous plasma, which would be expected to contain some cortisol, it is clear that demonstration of glucocorticoid effects at concentrations lower than those tested would not be possible without the use of sera from which the physiological concentrations of glucocorticoid had been previously removed. The low concentrations to which T lymphocyte colony formation is sensitive fall well within the range required to saturate the high-affinity cytoplasmic receptors for glucocorticoids (Lippman *et al.*, 1973), and the inhibition of colony production is antagonized by cortexolone, which competes with active glucocorticoids for these receptors.

In both primary and secondary cultures conditioned medium from PHA-stimulated lymphocytes (CM) completely abrogated the inhibitory effects of MPS both in terms of colony numbers and colony size. The active factor in CM is almost certainly T cell growth factor (TCGF, interleukin II) as we have observed that the CM used was able to maintain T cell blasts in proliferation for several weeks after cells had lost their responsiveness to PHA (Table 3) (Lotze, Strausser & Rosenberg, 1980; Zeevi, Chiu & Duquesnoy, 1980; Smith *et al.*, 1980). However, conditioned medium from MPS-treated cultures (MPS-CM) showed a markedly reduced capacity to support secondary colony formation. Similarly, the number of secondary colonies developing on MPS-treated cell feeder layers was also reduced, presumably as a result of inhibition of TCGF production. These findings show that the major site of action of MPS on human T cell growth is the inhibition of release of growth factors from accessory cells rather than a direct effect on proliferation of colony cells.

Similar findings have been reported by Gillis, Crabtree & Smith (1979) who demonstrated that dexamethasone inhibits lymphocyte proliferation by reducing TCGF activity in culture supernatants of lectin-stimulated mouse and human lymphocytes.

Experiments in which MPS was added to cultures at varying times showed that early addition of MPS was necessary to inhibit colony formation. These findings are similar to those of Webel & Ritts (1977) and Robertson *et al.* (1981) who found that early addition or preincubation of cultures with MPS was necessary to inhibit thymidine incorporation in lectin-stimulated cells. This finding is consistent with the hypothesis that MPS acts mainly by inhibiting TCGF production as it is known that TCGF production occurs predominantly during the early stages of culture (Gillis *et al.*, 1978). The alternative possibility, that MPS acts by preventing initial activation of colony cells or their subsequent proliferation in response to TCGF is unlikely since the effects of MPS were reversed by addition of CM (and presumably TCGF) to cultures and it has been reported by others that treatment with the glucocorticoid dexamethasone has no effect on the ability of lectin-treated T cells to respond to TCGF (Larsson, 1980).

The question arises as to the relevance *in vivo* of these *in vitro* effects of glucocorticoid hormone on proliferating human T cells. The lymphopenia which rapidly follows systemic administration of steroid is more readily attributable to sequestration of circulating lymphocytes than to any effect on their proliferation (Fauci & Dale, 1974). However, T cells, derived from the peripheral blood, proliferate in the vicinity of accessory cells such as macrophages in inflammatory foci *in vivo*. The phenomena observed here *in vitro* bear a strong analogy to this situation. On the basis of the results

presented here, we propose that the major action of glucocorticoid hormones on lymphocytes within reactive and inflammatory foci is to inhibit production of soluble growth factors by accessory cells; we have been unable to detect any direct effect on the proliferating T cells themselves.

A.S.K. is supported by an MRC Training Fellowship and A.H.W. by a grant and a Career Development Award from the Cancer Research Campaign. We are also grateful for grant support to the Lothian Health Board and the Melville Trust.

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Action of deoxycoformycin on human T cell colonies *in vitro*

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(Accepted for publication 14 May 1982)

SUMMARY

The potent adenosine deaminase inhibitor, deoxycoformycin (dCF), is currently under evaluation in the treatment of lymphoid malignancy. We show that dCF inhibits the growth in soft agar of T cell colonies from PHA stimulated human peripheral blood lymphocytes. In contrast to previous attempts to develop an *in vitro* model for analysis of the drug's action, concentrations lower than 10^{-9}M are effective, and no 'priming' by pharmacological concentrations of adenosine is required. Maximum inhibition is obtained when dCF is present over the first 4 hr of cellular exposure to PHA. T cells already proliferating in response to PHA are less sensitive to dCF, implying that S-phase events are not primary targets of the drug's action. Colony inhibition does not appear to be due to alteration in the production of, or sensitivity to, soluble T cell growth factors. In suspension cultures, dCF at concentrations up to 10^{-5}M fails to inhibit early PHA-induced volume changes, or later mitosis, in peripheral blood lymphocytes. The results show that there is a critical dCF sensitive step early in PHA stimulation. It involves those T cells capable of forming colonies and may be conditioned by the cellular microenvironment.

INTRODUCTION

The essential role of adenosine deaminase (ADA) in lymphocyte function was first identified by the description of severe combined immunodeficiency disease resulting from the congenital absence of the enzyme (Giblett *et al.*, 1972; Dissing & Knudsen, 1972). Deoxycoformycin (dCF) is a potent inhibitor of ADA (Woo *et al.*, 1974) that has been shown experimentally to mimic ADA deficiency by causing a selective lymphopenia in mice (Smyth, Young & Young, 1978). Early clinical trials have shown that dCF inhibits human lymphocyte ADA, is cytotoxic to malignant lymphocytes particularly of T cell lineage—cells known to have elevated ADA activity—and has therapeutic activity in T cell neoplasms (Smyth *et al.*, 1980; Poplack *et al.*, 1981; Prentice *et al.*, 1981). The lymphopenia produced by inhibition of ADA suggests that dCF might be useful as an immunosuppressant.

Despite a number of hypotheses, the precise metabolic role of ADA in normal lymphocyte function is uncertain. At the cellular level we do not know whether ADA inhibitors such as dCF produce pharmacological immunodeficiency as a result of a general suppression of all T cells or of some critical subpopulation. Nor is it clear whether the drug acts directly on proliferating T cells, on accessory cells involved in T cell activation, or through inhibition of the action of humoral factors such as the interleukins.

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0009-9104/82/1000-0115$02.00

This paper describes a cell culture system in which the cellular mode of action of dCF can be studied in some detail. The system, which involves growth of human T cells in soft agar (Riou *et al.*, 1976), provides a more sensitive assay of the drug's cellular actions than any whole cell *in vitro* method reported so far and allows analysis of the effects of cell interactions and differentiation on sensitivity to dCF.

MATERIALS AND METHODS

Mononuclear cells. These were obtained by sedimentation in Ficoll-Hypaque (Böyum, 1968) of heparinized venous blood from healthy young adult volunteers of either sex. Before culture, the cells were washed twice in RPMI 1640 medium (GIBCO), counted by Coulter counter, and assessed for 'viability' by nigrosine exclusion. In all experiments more than 95% cells excluded nigrosine. The plasma was retained and used to supplement the culture medium.

Primary lymphocyte cultures. Cultures in soft agar were established by a modification of the method of Riou *et al.* (1976) as already described (Krajewski & Wyllie, 1981). In brief, the mononuclear cells were suspended at concentrations between 5 and 20×10^6/ml in 0·33% agar (DIFCO) in RPMI 1640 supplemented with 7·5% autologous plasma. This cell suspension was pipetted on to the surface of an underlay of 0·5% agar in RPMI 1640 supplemented with 1% phytohaemagglutinin M (DIFCO) (PHA) and 15% autologous plasma. In both underlay and cell suspension, the RPMI 1640 included 80 μg/ml streptomycin and 80 iu/ml penicillin G (crystamycin, Glaxo). Cultures were set up in 16 mm wells of 'Linbro' multiwell plates (Flow Laboratories), using 220 μl underlay and 40 μl cell suspension. After incubation for 5 days at 37°C in a humidified atmosphere of 95% air/5% CO_2, colonies were counted in transmitted light using a microscope with a × 10 objective. Colonies were defined as aggregates of more than about 40 cells. In each well the total colony count from 10 fields was recorded. Three or more wells were counted for each culture condition in each experiment.

Secondary soft agar cultures. These were established by a modification of the method of Goube de la Forest *et al.* (1979). T cell 'blasts' were obtained from primary cultures in 90 mm petri dishes, each of which contained 6·8 ml underlay and 1·2 ml cell suspension as above. After 6 days' primary culture the cells were harvested, washed in RPMI 1640 and suspended at $7{\cdot}5 \times 10^6$ cells/ml in 0·33% agar as above. Forty microlitre aliquots of this suspension were pipetted on to the surface of underlays in Linbro plate wells. The underlay in these secondary cultures differed from that in the primary cultures in that PHA was omitted, and conditioned medium (as described below) was added at a final concentration of 25–50%.

Conditioned media. These were prepared from suspensions of peripheral blood mononuclear cells (10^6 cells/ml) in RPMI 1640 supplemented with 1% autologous plasma and 1% PHA. Three millilitre aliquots of these suspensions were incubated in 25 ml Falcon flasks for 48 hr. Cells and cellular debris were removed by centrifugation at 700 ***g*** for 20 min. The supernatants were stored at −20°C and used within 1 month. Ability of conditioned media to support DNA synthesis in T cell 'blasts' was measured as follows. Cells harvested from primary agar cultures were suspended at 10^5/ml in RPMI 1640 supplemented with 10% newborn calf serum. One fifth of a millilitre of this suspension was incubated with 0·2 ml of a test solution in Linbro plate wells. Test solutions comprised RPMI 1640 alone, RPMI 1640 containing 1% PHA, or conditioned media. After 4 days' incubation at 37°C the wells were pulsed with 1 μCi ^{3}H-thymidine (Amersham) for 4 hr. Cells were collected on Whatman GF/c filters, washed twice with ice cold phosphate-buffered saline (PBS), precipitated by three washes of 5% trichloroacetic acid, and rinsed with absolute ethanol. The filters were then dried and counted in scintillant, with an efficiency for tritium of around 50%. All conditioned media prepared from untreated cells stimulated thymidine incorporation 4–10-fold more than could be attributed to the residual presence of PHA alone.

Deoxycoformycin. Freeze dried 2′ deoxycoformycin ('Pentostatin', Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland, USA) was reconstituted with PBS, resterilized by filtration and added to cultures by pipetting on to the surface of the agar. Control cultures received the same volume of saline. Pilot experiments showed that dCF was much less

effective when incorporated into the agar underlay than when pipetted on to the surface. Application of dCF to the agar surface had the further advantage that it could be done at any time after setting up the culture. It is however difficult to be precise about drug concentrations reaching cells by diffusion through soft agar. Cited concentrations are those pipetted on to the agar surface and are therefore all over-estimates by up to 10-fold of the concentrations surrounding the cells, since the volume of saline in which the dCF was applied was one tenth that of the agar in each well.

Methods for cell identification. These included sheep RBC rosetting and indirect immunofluorescence for surface immunoglobulins (Habeshaw & Young, 1975), in cell suspensions. Cytoplasmic immunoglobulin and non-specific esterase activity (Yam, Li & Crosby, 1971) were detected in ethanol fixed cytospin preparations.

Assessment of PHA transformation in suspension cultures. In some experiments, the effect of dCF was studied on peripheral blood lymphocytes undergoing PHA transformation in suspension culture. As it is theoretically possible that ^{3}H-thymidine uptake measurement would be misleading following dCF treatment, because of alterations in the deoxyribonucleoside pool (Smyth & Harrap, 1977), PHA-induced transformation was assessed by cell volume spectroscopy (to monitor early changes) and by scoring mitotic indices (to monitor late changes).

Volume spectroscopy. This was conducted by the method of Gibbs *et al.* (1979) after 22 hrs exposure of lymphocytes at a concentration of 2×10^6/ml to PHA. This method quantifies the well known increase in size of PHA stimulated lymphocytes in the first 24 hr of transformation, prior to entry to S-phase. Size distributions of stimulated and control cell populations are obtained by Coulter counter analysis and permit derivation of two scores: the percentage of cells showing a cell size increase (% growing cells) and the rate at which this size increase takes place (incremental growth rate). dCF was present in the treated cultures throughout the incubation, at concentrations between 10^{-9} and 10^{-5} M.

Cells for morphological assessment of mitotic index. These were cultured for up to 72 hr at 10^6/ml in sterile Wasserman tubes, in the presence of PHA and, in treated cultures, dCF at a final concentration of 10^{-7} M. Cells were collected by centrifugation (110 ***g*** for 10 min), smeared on glass slides, fixed in Bouin's fluid for 15 min and stained by the Feulgen reaction. The proportion of mitotic cells was assessed from examination of 500 consecutive cells on each smear.

RESULTS

dCF inhibits colony formation at low concentrations

Within 3–4 days of incubation, the untreated cultures included many small aggregates of cells on or immediately below the agar surface. By 5–6 days, these had grown into large groups of closely packed cells. The great majority of these cells displayed T cell characteristics: 80–90% formed rosettes with sheep red blood cells, whereas only 5% possessed surface immunoglobulin, 3% cytoplasmic immunoglobulin and 0·01% non-specific esterase. When dCF was added at 10^{-9} M at the onset of the incubation, fewer colonies grew than in untreated controls. The reduction in colony numbers was dose related up to about 10^{-6} M (Fig. 1). In the colonies which did develop, however, the proportion of cells showing T, B, plasma cell and monocyte markers were exactly as above.

By contrast dCF had little effect on PHA-induced changes in peripheral blood lymphocytes incubated in free suspension. Thus in concentrations up to 10^{-5}M dCF had no effect on the PHA-induced increase in % 'growing cells' or on the incremental growth rate, and there was no difference in the mitotic indices in smears of suspension cultures incubated without dCF or with dCF at 10^{-7} M (Table 1).

dCF is maximally effective early after PHA stimulation

The inhibition of colony formation by dCF might be due to an action of the drug on T cell proliferation in general, or to a more restricted action on the cellular events responsible for the initiation of colonies. To distinguish between these possibilities, dCF was added to primary cultures

at various times after their initiation or was incubated briefly with the peripheral blood lymphocytes before culture was initiated (Table 2). dCF was most effective in inhibiting colony formation when present during the first 4 hrs of culture. If added thereafter, despite its presence in the cultures over the next 5 days, colony inhibition was much less. Only slight inhibition of colony formation was observed when cells were exposed briefly to dCF prior to incubation in soft agar and PHA (Table 2).

Further, cells from previously untreated colonies were harvested and tested for responsiveness to dCF in secondary culture. Such cells proliferate to form new colonies provided they are supplied with humoral factors present in conditioned media. dCF did not significantly inhibit the number of these new colonies developing in medium, supplemented with 50% conditioned medium, prepared as described in Methods. When the conditioned medium supplement was halved, colony numbers in untreated and dCF treated cultures were both reduced, and to the same degree.

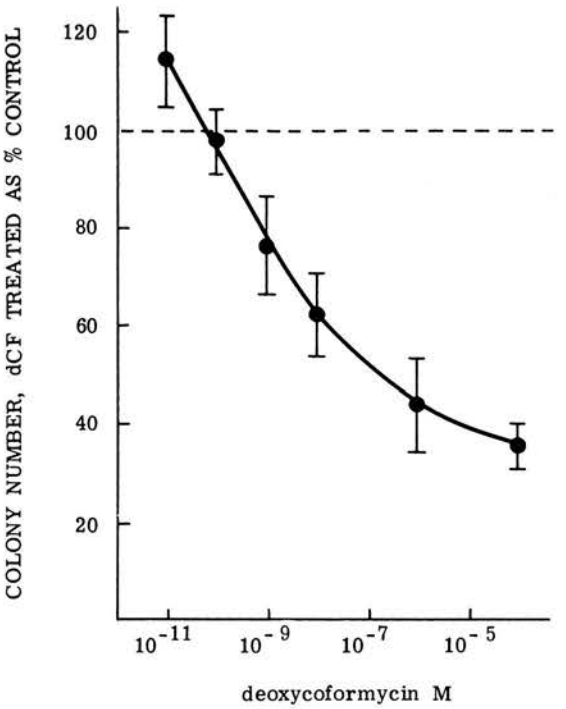

Fig. 1. Effect of dCF on T cell colony growth *in vitro*. Results are the means ± 1 s.e. of between six and eight replicate cultures. Colony numbers are significantly less than control in cultures treated with dCF applied at concentrations of 10^{-9} M ($P < 0{\cdot}05$, *t*-test), 10^{-8} M ($P < 0{\cdot}005$), 10^{-6} M and 10^{-4} M ($P < 0{\cdot}0005$).

dCF does not affect interleukin production by PHA stimulated lymphocytes

It is possible that dCF might inhibit T cell colony formation indirectly, through inhibition of production of humoral T cell growth factors. To test this hypothesis, conditioned media derived from cultures of control or dCF treated, PHA stimulated mononuclear cells, were assayed for their ability to support the growth of T cell colonies in secondary culture. There were no significant differences in the number of colonies which developed in the presence of conditioned media from control or dCF treated cells and similar numbers of colonies developed when dCF was added directly to the assay culture (Table 3).

Table 1. Effect of dCF on PHA-induced transformation of peripheral blood lymphocytes in suspension culture

*(a) Incremental growth rate and percentage growing cells over first 18 hr**

dCF (M)	Incremental growth rate mean (±s.e.) $\times 10^2$	% growing cells mean (±s.e.)
0	4·88 (±0·18)	90·42 (±1·16)
10^{-9}	4·63 (±0·16)	87·10 (±3·14)
10^{-8}	4·55 (±0·22)	87·22 (±4·48)
10^{-7}	4·73 (±0·24)	86·56 (±3·77)
10^{-6}	5·00 (±0·15)	90·46 (±1·53)
10^{-5}	5·02 (±0·08)	91·12 (±0·50)

(b) Mitotic indices (mean±s.e.)†

	Time since addition of PHA		
	24 hr	48 hr	72 hr
Without dCF	0·00	0·07±0·07	3·37±0·92
With dCF (10^{-7} M)	0·13±0·13	0·27±0·13	5·20±1·37

* Each result is the mean (±1 s.e.) of at least five observations. 'Incremental growth rate' and '% growing cells' were derived from Coulter counter analysis as described in Methods.

† Each result is the mean (±1 s.e.) of three experiments from each of which 500 cells were scored. None of the differences between dCF treated and control cells is significant.

Table 2. Effects of dCF on T cell colony formation when added at various stages in T cell response to PHA

Culture conditions	No. of experiments	Colony number (mean±s.e. as % control scored after 5 days)
dCF incubated with cells for 2 hr prior to PHA	5	81·7±4·7
dCF added to primary cultures in PHA after		
0 hr	5	63·2±3·8
2 hr	5	56·3±1·2
4 hr	5	86·0±4·4
8 hr	5	82·3±4·4
24 hr	3	82·2±4·0
48 hr	3	96·8±1·4

dCF was added at a concentration of 10^{-6} M. Colony number is significantly less when dCF was added at 0 hr and 2 hr than when it was added at 4 hr ($P < 0{\cdot}0025$); the depression in colony number when dCF was added at other times up to 24 hr is also significant, although less profound ($0{\cdot}005 < P < 0{\cdot}05$).

Table 3. Effect of dCF on growth factor production

Culture conditions	Colony number (mean ± s.e.)
CM* from untreated cells	68·6 ± 9·4
CM* from dCF treated cells	52·0 ± 11·2
CM* from untreated cells; dCF added to assay cultures	66·9 ± 11·2

CM* = cell free conditioned medium, included in assay culture agar to 50% volume.

Results are from four separate experiments. There are no significant differences in colony number between these three conditions.

DISCUSSION

We have demonstrated that certain peripheral blood T cells—those which are the progenitors of T cell colonies *in vitro*—are sensitive to dCF at concentrations lower than 10^{-9} M. This is well within the concentration achieved in plasma during the treatment of patients, and associated with profound lymphopenia (Smyth *et al.*, 1980). It is relevant that the dissociation constant of the reaction of dCF with adenosine deaminase extracted from human erythrocytes is about 10^{-11} M (Agarwal, Spector & Parks, 1977).

Our method employs lymphocytes cultured in semi-solid agar. The results contrast with previous studies on the consequences of ADA inhibition *in vitro*, in which lymphocytes in suspension culture were exposed to both deoxycoformycin and the ribose equivalent, coformycin. PHA transformation in suspension culture is inhibited to a variable extent (Hovi *et al.*, 1976) or not at all (Snyder, Mendelsohn & Seegmiller, 1976; Carson & Kaye, 1979; Uberti, Lightbody & Johnson, 1979) by concentrations of coformycins many orders of magnitude greater than 10^{-9} M. Using methods which detect both early and late phases of PHA transformation, and which are independent of any dCF related alterations in nucleoside pools, we have confirmed this insensitivity of lymphocytes in suspension culture. Previous workers have, however, observed inhibition when the lymphocytes were exposed concurrently to high concentrations of adenosine.

The high sensitivity of human peripheral blood T cells to dCF recorded in this study, in which adenosine was not added to the culture media, may be attributable to two factors. Firstly, certain cell derived substances (perhaps including adenosine) may reach higher concentrations in the immediate microenvironment of cells in soft agar than are attained around cells in free suspension. Secondly, the soft agar method preferentially studies a numerically very small subpopulation of peripheral blood T cells, which is maximally sensitive to dCF immediately after PHA stimulation. The proliferating T cells which are the product of this stimulation are less sensitive to dCF, yet it is these cells which have usually been studied in attempts to analyse the drug's actions.

The question arises as to the mechanism of this high sensitivity to dCF early after PHA stimulation. The period of maximum sensitivity recorded here occurs around 20 hr prior to the onset of DNA synthesis in PHA stimulated cells observed by several workers (Jasinska, Steffen & Michalowski, 1970; Soren, 1973; O'Leary *et al.*, 1980), implying that events within S-phase are not the primary target of the drug. These results concord with those of Uberti *et al.* (1979) who demonstrated that lymphocytes in suspension culture, if supplemented with adenosine, are maximally sensitive to dCF early after PHA stimulation. It is possible that restriction in the production of DNA synthesis precursors may account for this (Cohen *et al.*, 1978), but colony forming T cells in secondary culture appear relatively insensitive to dCF although actively engaged in DNA synthesis. Moreover adenosine deaminase inhibition has been shown to exert lethal effects on those cells from the human thymus which do not engage in DNA synthesis (Kefford & Fox,

1982). Others have suggested that the effects of ADA inhibition are due to intracellular accumulation of cAMP (Wolberg *et al.*, 1975; Carson & Seegmiller, 1976) or to inhibition of methylation reactions (Kredich & Martin, 1977). The role—and indeed the existence—of early changes in cAMP concentration in PHA-induced lymphocyte transformation is still uncertain (reviewed by Hume & Weidemann, 1980), but histone methylation has been demonstrated within the first hour of exposure to PHA (Monjardino & MacGillivray, 1970).

A different class of mechanism would be an effect of dCF on accessory cells involved in the cellular interactions known to be essential in lymphocyte transformation (summarized in O'Brien, Parker & Dixon, 1978) and to occur within the first few hours of PHA stimulation. Some of these interactions are mediated by soluble factors such as the interleukins (Smith *et al.*, 1980). We did not detect inhibition by dCF of the production of such soluble factors, however, using an assay system sufficiently sensitive to demonstrate low concentrations of a glucocorticoid which does act in this way (Krajewski & Wyllie, 1981). The secondary colony assay measures T cell growth factor (interleukin-2) activity. However, release of this into the conditioned medium by cultured mononuclear cells is dependent upon their production of interleukin-1. The failure of dCF to diminish the potency of such conditioned medium thus makes it unlikely that dCF exerts its inhibitory effects through modification of either of these soluble factors. Further, the resistance to dCF of colonies growing in secondary culture, supported by conditioned media, makes it improbable that dCF reduces the sensitivity of colony forming cells to such factors. We have not excluded, however, the possibility that the dCF sensitive event might involve a direct physical interaction between colony forming T cells and accessory cells.

In conclusion, the simple, *in vitro* culture system described has made it possible to identify an effect of dCF on lymphocyte proliferation at concentrations of the drug known to be pharmacologically effective *in vivo*, but without additions of nucleoside to the culture medium. The data show that there is a step in T cell blastogenesis which is highly dCF sensitive, and precedes the onset of DNA synthesis by many hours. More precise definition of the dCF sensitive event is desirable, as it will shed light not only on the mode of action of this promising new chemotherapeutic agent, but also on the role of ADA in processes critical to the initiation of lymphocyte proliferation and differentiation.

We are grateful to Professor J. Swanson Beck, Department of Pathology, Dundee University, for his help with the cell volume spectroscopy work. ASK is supported by a Research Training Fellowship from the Medical Research Council, AHW by a grant and Career Development Award from the Cancer Research Campaign, and NC by a scholarship from the Scottish Home and Health Department.

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Colony formation and interleukin 2 production by leukaemic human T cells

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(Accepted for publication 20 May 1983)

SUMMARY

PHA-induced colony formation and interleukin 2 (IL-2) production were studied in four patients with T cell leukaemia (three cases OKT4$^+$/T helper and one case OKT8$^+$/T cytotoxic suppressor). Cases of T helper cell leukaemia showed colony formation that was comparable to normal purified blood T cells and was not dependent on the addition of conditioned medium, containing IL-2 activity, to cultures. In contrast the T suppressor cell leukaemia formed colonies only when cultures were supplemented with IL-2 containing medium. When IL-2 production by PHA stimulated cells was measured culture supernatants from the three T helper cell leukaemias all showed normal or high levels of activity, when compared to normal blood mononuclear cells, whereas the T suppressor cell leukaemia showed no activity.

Keywords T cell leukaemia colonies interleukin 2

INTRODUCTION

Recently, leukaemic cells in adult chronic T cell leukaemia have been shown to express differentiation markers characteristic of mature T lymphocytes and have been classified as either T helper or T suppressor–cytotoxic cell tumours on the basis of the cell surface phenotype determined using monoclonal antibodies such as OKT4 and OKT8 (Reinherz & Schlossman, 1981; Foon, Schroff & Gale, 1982). Relatively little, however, is known of the function of these cells and to what extent these leukaemic cells produce or respond to the growth factors (interleukins) that regulate normal T cell proliferation (Foa & Catovsky, 1979; De Vries, Vyth & Mendelsohn, 1981; Friedman *et al.*, 1982; Palacios, 1982).

In order to investigate these problems we have studied PHA-induced interleukin-2 (IL-2) production and T cell proliferation in four patients with chronic T cell leukaemia. T cell proliferation was measured using an *in vitro* colony forming assay in soft agar (Krajewski & Wyllie, 1981). By measuring colony formation in cultures supplemented with interleukin 1(IL-1) and IL-2 the growth factor responsiveness of normal and leukaemic T cells could be compared.

In this report we show that leukaemic cells with a T helper phenotype produce IL-2 and proliferate to form colonies in response to PHA. Colony formation by leukaemic T helper cells was enhanced by both IL-1 and IL-2. In contrast leukaemic cells with a T suppressor–cytotoxic phenotype failed to produce IL-2 or proliferate in response to PHA and IL-1 but did form colonies in the presence of IL-2.

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MATERIALS AND METHODS

Preparation of cell suspensions. Blood mononuclear cells (MC) from normal individuals and patients with T cell leukaemia were isolated from heparinized venous blood by centrifugation over Ficoll-Hypaque. Spleen mononuclear cells were obtained from patients who had undergone splenectomy for congenital spherocytosis. Spleen cells were obtained by scraping the cut surface of spleen in heparinized tissue culture medium, followed by sieving through a fine wire mesh and centrifugation over Ficoll-Hypaque. MC suspensions from the plasma-Ficoll interface were washed twice in RPMI 1640 and then resuspended at the appropriate cell concentration. In all cases cell viability, assessed by trypan blue dye exclusion, was greater than 95%.

T cell enriched MC suspensions were prepared by rosetting blood MC with neuraminidase treated sheep red blood cells followed by centrifugation over Ficoll-Hypaque. Sheep red cells in the cell pellet were removed by hypotonic lysis and the purified T cells resuspended in culture medium.

Colony formation. Colony formation by blood MC was assessed as previously described (Krajewski & Wyllie, 1981) with some modifications. Blood MC were suspended at 5×10^6 cells/ml in 0·33% agar in RPMI 1640 containing 15% heat-inactivated human serum. Forty microlitres of this suspension (2×10^5 cells) was pipetted on to underlayers of 0·2 ml of 0·5% agar in RPMI 1640 containing 15% human serum, 20 μg PHA-M (DIFCO) and 0·5% washed human red blood cells. Cells were also cultured on underlayers supplemented with spleen lymphocyte conditioned medium (25%) or monocyte conditioned medium (15%). Cultures were carried out in 17 mm diameter wells in multiwell dishes (Linbro 76-033-05).

Colonies, defined as cell aggregates greater than 40 cells, were scored after 5–6 days culture, at 37°C in a fully humidified atmosphere of 5% CO_2 in air. Scoring was carried out by counting three fields per well at ×40 magnification using a ruled eye piece grid. Each grid area represented approximately 1/20 of total well area. The total number of colonies per well was estimated. Thus

$$\frac{\text{number counted in 3 grids} \times 20}{3}.$$

Conditioned media. Monocyte conditioned medium (MoCM) was prepared as described by Lachman, Moore & Metzgar (1978). Human monocytic leukaemia cells were incubated at $0{\cdot}5 \times 10^6$ cells/ml in RPMI 1640 supplemented with 5% heat-inactivated human serum and 10^{-5}M 2-mercaptoethanol. After 24 h incubation at 37°C in 5% Co_2 in Roux flasks, the supernatants were collected, centrifuged at 800*g* for 20 min, aliquoted, frozen and stored at −20°C until used. MoCM was shown to contain IL-1 activity by enhancement of human thymocyte mitogenesis (Maizel *et al.*, 1981) with maximum activity when present at 15% concentration. MoCM did not contain IL-2 activity, as assayed below.

Lymphocyte conditioned medium (LyCM) was prepared by incubating human spleen cells at 1×10^6 cells/ml in RPMI 1640, with 1% PHA, 1% human serum and 1 μg/ml indomethacin (Inouye *et al.*, 1980). Supernatants were harvested after 48 h incubation and stored at −20°C until used. These supernatants contained IL-2 activity as assayed below.

IL-2 assay. Culture supernatants from PHA stimulated MC (prepared as described for LyCM) were tested for IL-2 activity using techniques similar to those described by Gillis *et al.* (1978). Target cells for testing IL-2 activity were T colony cells harvested from primary cultures after 6–7 days (Krajewski & Wyllie, 1981). These cells do not respond to PHA but proliferate if LyCM is present. T colony cells were suspended at 1×10^5 cells/ml in RPMI 1640 with 20% heat-inactivated human serum and 0·1 ml aliquots were added to 10 mm diameter flat bottomed wells containing 0·1 ml of test supernatant. Duplicate cultures of supernatants at concentrations between 50 and 6% were assayed. After 96 h incubation at 37°C cultures were pulsed for 4 h with 0·2 μCi of ^{3}H-thymidine per well and then harvested on to filters using an automatic harvester (Multimash Cell Harvester, Dynatech Laboratories Ltd.). The cells were washed with 0·85% NaCl and then with 5% TCA, the filters dried and placed in scintillant for measurement of acid precipitable ^{3}H-thymidine incorporation.

Cell phenotyping. Rosetting with sheep red blood cells (E rosettes) and mouse red cells (MRBC

rosettes) and staining for acid phosphatase and non-specific esterase were carried out as previously described (Dewar, Krajewski & Murray, 1980; Stockdill, Dewar & Harrison, 1983).

Lymphocyte subsets were identified using monoclonal anti-T cell antibodies, OKT3, OKT4, OKT8 (Ortho Diagnostics Ltd.) and the anti-HLA-DR antibody DA6-231 (Guy *et al.*, 1983). Aliquots of $0{\cdot}5$–$1{\cdot}0 \times 10^6$ cells were pelleted in 12×7 mm glass tubes and the supernatants removed. Ten microlitres of monoclonal antibody were added to the appropriate tube and cells resuspended and incubated on ice for 30 min. Cells were then washed twice in ice cold PBS and the supernatants drained off. Twenty microlitres of FITC conjugated goat anti-mouse immunoglobulin (FITC-GAMIg) (Meloy Ltd.) was added and suspensions incubated for 30 min on ice and then washed twice. A drop of cell suspension was placed on a slide under a cover slip and 200 cells were examined for fluorescence. Controls stained with FITC-GAMIg only were also examined. The controls always showed less than 5% positive (i.e. non-specific staining) cells.

RESULTS

Cases studied

Four patients with chronic T cell leukaemia were studied. In cases 1–3 blood mononuclear cells showed a T helper phenotype ($OKT3^+$, $OKT4^+$, $OKT8^-$) and in case 4 a T suppressor–cytotoxic phenotype ($OKT3^+$, $OKT4^-$, $OKT8^+$). The leukaemic cells also showed cytochemical staining typical of T cells (focal acid phosphatase and non-specific esterase positivity). All cases were negative for Tdt, and cells did not express B cell markers (HLA-DR, mouse red blood cell receptor).

Morphologically cases 2 and 3 were classified as lymphocytic leukaemias and cases 1 and 4 as prolymphocytic leukaemias (Table 1).

Colony formation

Colony formation was measured on underlayers containing either PHA alone or supplemented with monocyte conditioned medium, as a source of IL-1, or lymphocyte conditioned medium, as a source of IL-2. The three cases of T helper cell leukaemia (cases 1–3) showed colony formation that was comparable to that of purified normal blood T cells. Colony formation in these cases was enhanced by both monocyte and lymphocyte conditioned media. In contrast in the case of the T suppressor–cytotoxic leukaemia (case 4) colonies developed only when cultures contained lymphocyte conditioned medium (Table 2).

Table 1. Cell phenotype in four cases of chronic T cell leukaemia

			% positive mononuclear cells							
Patient	Age/sex	WCC	%lyms	OKT3	OKT4	OKT8	HLA-DR	E	MRBC	Diagnosis
1	73 M	213	98	98	97	5	1·5	85	2	T helper prolymphocytic leukaemia
2	82 F	51·5	97	96·5	100	0·5	—	77·5	0·5	T helper lymphocytic leukaemia
3	65 F	47	85	81	100	1·0	1·0	17	1·0	T helper lymphocytic leukaemia
4	49 M	15	95	94	25*	88·5	1·0	79·5	6·5	T suppressor/cytotoxic prolymphocytic leukaemia
Normal range				46–70	33–64	16–32	15–30	43–68	0–5	

* (weak reaction).

All cases were Tdt negative. Cytochemical staining showed strong focal acid phosphatase and non-specific esterase activity in cases 1–3 and strong acid phosphatase but weak non-specific esterase activity in case 4.

Table 2. Colony formation by normal and leukaemic cells

	Colonies/2×10^5 cells						
	Normals (4)*			T cell leukaemia			
Culture underlayer	MC	E^+	(range)	1	2	3	4
PHA	1,026	133	(0–267)	80	213	813	0
PHA + monocyte CM (IL-1)†	1,140	1,320	(687–2,233)	567	1,500	1,687	0
PHA + lymphocyte CM (IL-2)‡	1,167	1,440	(840–1,913)	853	1,680	1,687	480

* Blood mononuclear cells (MC) contained 47–68% T cells (mean 56% E^+) and 10–13% monocytes (non-specific esterase positive cells). T cells (E^+) purified by rosetting with sheep RBC contained 93–98% T cells (mean 96% E^+) and less than 1% monocytes. Figures for normals are mean values of experiments on four different donors. Figures for leukaemics are mean values of triplicate cultures in a single experiment.

† Monocyte CM (IL-1) was prepared by culturing human monocytic leukaemia cells at $0{\cdot}5 \times 10^6$/ml in RPMI 1640 with 5% human serum and 10^{-5}M 2-mercaptoethanol for 24 h. The supernatant from these cultures enhanced thymocyte mitogenesis but was unable to stimulate proliferation of cultured T cells.

‡ Lymphocyte CM (IL-2) was prepared by culturing human spleen cells at 1×10^6/ml in RPMI 1640 with 1% PHA, 1% serum and 1 μg/ml indomethacin for 48 h. The supernatant from these cultures was able to stimulate proliferation of cultured T cells that were no longer responsive to PHA alone.

The colonies that developed in case 4 were smaller than in cases 1–3 and showed early degeneration, whereas colonies in cases 1–3 continued to enlarge without degenerative changes occurring over the 6–7 day culture period.

Colony cells harvested from agar cultures were morphologically large, transformed cells some of which were in mitosis. Monoclonal antibody analyses showed that these expressed a similar phenotype to the initial T cell population, i.e. colony cells derived from normal blood mononuclear cells consisted of a mixture of $OKT4^+$ and $OKT8^+$ cells, whereas colony cells from leukaemics showed a predominance of either $OKT4^+$ cells (cases 1–3) or $OKT8^+$ cells (case 4) (Table 3).

IL-2 production

In parallel with the colony forming assays, experiments were performed to determine whether leukaemic T cells were able to produce IL-2. Supernatants from PHA stimulated cell cultures were tested for IL-2 activity by assessing their ability to stimulate proliferation of normal cultured T cells which were not responsive to PHA. Supernatants from the three cases of T helper cell leukaemia all showed normal or high levels of IL-2 activity, when compared to supernatants from normal blood

Table 3. Phenotype of colony cells

	% positive cells			
Patient	OKT3	OKT4	OKT8	E rosettes
1	97	93	0	99·5
2	93	92	0	99·5
3	72	85	3	93
4	—	10	43	—
Normals (4)	93–98·5	42·5–78	34–52	93–99·5

Table 4. IL-2 production by normal and leukaemic cells

% Concentrations of conditioned medium		IL-2 activity (ct/min) 50%	25%	12%	6%
Case 1 (OKT4$^+$)	Expt 1	11,200	6,856	4,150	1,755
	Expt 2	11,385	4,516	4,916	2,042
	Expt 3	—	8,854	6,999	3,098
	Expt 4	10,673	8,652	4,299	2,451
Case 2 (OKT4$^+$)	Expt 1	12,058	13,407	11,492	9,353
	Expt 3	12,837	11,634	9,250	8,107
Case 3 (OKT4$^+$)	Expt 2	13,240	4,803	13,555	10,538
	Expt 4	18,843	16,821	15,103	9,522
Case (OKT8$^+$)	Expt 2	1,027	1,645	984	286
	Expt 4	735	445	496	286
Normal blood MC	Expt 1	9,192 ± 2,887	7,123 ± 4,108	4,771 ± 3,057	2,240 ± 1,184
Spleen MC (LyCM)	Expt 1	11,573	5,965	3,821	3,120
PHA control	Expt 1	1,400	482	906	1,105
	Expt 2	1,035	1,830	582	705
	Expt 3	1,547	748	682	837
	Expt 4	115	—	—	—

Conditioned media from PHA stimulated MC were prepared and tested for IL-2 activity as described in Materials and Methods. Figures for normal MC are the mean value ± 1 s.d. of IL-2 activity in conditioned medium from eight different normal individuals. In each experiment a PHA control solution (1% PHA in RPMI 1640) was tested at the given dilutions. Responses to RPMI 1640 alone were 330 ct/min (Expt 1), 687 ct/min (Expt 2), 637 ct/min (Expt 3) and 225 ct/min (Expt 4).

mononuclear cells. The T suppressor-cytotoxic cell leukaemia showed no evidence of IL-2 production (Table 4).

DISCUSSION

Colony formation by normal T cells is known to depend upon interactions between colony forming cell precursors and accessory cells that modulate colony formation through the release of T cell stimulatory or inhibitory factors (Rosenszajn *et al.*, 1981; Klein *et al.*, 1982). Thus colony formation depends not only on the presence of growth factor responsive precursors, but also on the amount of growth factor produced by cultured cells or added to cultures (Krajewski & Wyllie, 1981).

In the present study we have shown that colony formation can be induced in leukaemic T cells by PHA and shows a similar growth factor dependency to that of normal T cells. T helper (OKT4$^+$) cell leukaemias formed similar numbers of colonies to normal human T cells and, like normal T cells, required IL-1 for optimal colony formation (Claesson *et al.*, 1977; Rosenszajn *et al.*, 1981). Colony formation by the T suppressor-cytotoxic (OKT8$^+$) cell leukaemia however differed from that of normal T cells in that colony formation could only be induced when IL-2 containing conditioned medium was added to cultures.

The differences in the culture requirements of OKT4$^+$ and OKT8$^+$ leukaemic T cells could be explained by differences in the ability of the leukaemic cells to produce IL-2. In the present study we have shown the OKT4$^+$ leukaemic cells produced IL-2 in response to PHA, but the OKT8$^+$ leukaemic cells did not. These findings are consistent with the observation that IL-2 production by normal T cells is a function of the OKT4$^+$ T cell subset (Reinherz *et al.*, 1980; Palacios, 1982), and

suggest that leukaemic cells expressing helper T cell phenotypes retain some of the functional capabilities of their normal counterparts. These findings are consistent with two previous case studies of chronic T cell leukaemia which have shown that $OKT4^+$ leukaemic cells produce IL-2 (Friedman *et al.*, 1982; Gramatzki *et al.*, 1982). Investigations of IL-2 production by $OKT8^+$ leukaemic T cells have not been reported; however De Vries *et al.* (1981) have described a case of T-CLL that responds to but does not produce IL-2. Although this case was not phenotyped using monoclonal antibodies this may be similar to our case of $OKT8^+$ leukaemia.

While it is tempting to postulate that autostimulation by IL-2 producing and responsive cells may, in some cases, play a role in leukaemic cell proliferation *in vivo*, we agree with Gootenberg *et al.* (1981) that this is unlikely to be a key event in induction of T cell neoplasia. The clinical significance of differences in T cell phenotype and function in chronic T cell leukaemia is at present uncertain. It is possible, however, that these differences, once accurately defined, may allow not only more precise classification of clinico-pathological groups but may also be utilized to control proliferation of the neoplastic cells.

This work was supported by a grant from the Scottish Hospitals Endowment Research Trust. The authors are grateful to Dr A.H. Wyllie for advice and support and to Miss Margo Wightman and Mrs Fiona Govan for preparation of the manuscript. We thank Dr A.C. Parker in the Haematology Department of the Royal Infirmary, Edinburgh for carrying out Tdt estimations. The monoclonal antibody DA6 231 was provided by Dr K. Guy.

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Resistance to methylprednisolone in cultures of blood mononuclear cells from glucocorticoid-resistant asthmatic patients

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(*Received 15 February/21 May 1984; accepted 29 June 1984*)

Summary

1. In order to investigate the cellular mechanism of glucocorticoid resistance in chronic asthma, peripheral blood mononuclear cells (MNC) from asthmatic patients were cultured in soft agar.

2. Cells from patients known to be clinically sensitive to glucocorticoid therapy did not differ significantly from those of clinically resistant patients in terms of their immunophenotype or the number of colonies generated by culture in the presence of phytohaemagglutinin.

3. The glucocorticoid methylprednisolone (MP) at low concentration (10 nmol/l) inhibited colony growth from cells of glucocorticoid-sensitive patients, whereas there was much less inhibition of colony growth from resistant patients' cells.

4. In a small prospective study inhibition of colony growth by methylprednisolone *in vitro* correlated with the subsequently determined sensitivity of the patients' asthma to glucocorticoid therapy.

5. Assessment *in vitro* of glucocorticoid sensitivity may help to predict which patients may be spared ineffectual glucocorticoid medication. The results raise the possibility that peripheral blood mononuclear cells may respond to glucocorticoid in a similar manner to cells involved in the pathogenesis of asthma.

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Key words: asthma, glucocorticoid resistance, methylprednisolone, mononuclear cells, T-cell colony.

Abbreviations: MNC, mononuclear cells; MP, methylprednisolone sodium succinate.

Introduction

Some patients with chronic asthma are resistant to systemic treatment with glucocorticoids, even in high dosage. Measurements of forced expiratory volume in 1 s ($FEV_{1.0}$) show striking differences between the response of resistant and sensitive patients to the short-term administration of systemic glucocorticoids, in sharp contrast to the considerable increases recorded in both groups after use of a bronchodilator aerosol [1]. Detailed study of a large group of glucocorticoid-resistant asthmatic patients in Edinburgh has already identified some differences in the aetiology and clinical patterns of asthma between the two groups. It was considered improbable, however, that these differences were central to the phenomenon of glucocorticoid resistance. Only one of a wide range of laboratory investigations discriminated between resistant and sensitive asthmatic patients, the measurement of monocyte complement receptors and their enhancement by casein, a monocyte chemotactic factor [2]. In this paper we show that peripheral blood mononuclear cells (MNC) from glucocorticoid-resistant asthmatic patients are relatively unresponsive to glucocorticoids *in vitro* and we suggest that this may be used to predict glucocorticoid resistance

in asthmatic subjects whose clinical response to these drugs has not yet been assessed.

Patients and methods

Patients

The laboratory studies reported in this paper were performed on venous blood samples obtained from 41 patients with chronic asthma and 15 normal subjects (Table 1).

All 41 asthmatic patients had been shown to respond to the inhalation of an aqueous β_2-agonist aerosol (salbutamol, 5 mg) by an increase in the $FEV_{1.0}$ of at least 30% from the baseline value of less than 60% of the predicted normal value. This evidence of reversible airflow limitation was regarded as consistent with the diagnosis of asthma. The patients were, however, selected in such a way as to form three separate groups.

Group 1: 15 asthmatic patients who were 'glucocorticoid resistant' in that their $FEV_{1.0}$ did not increase by more than 15% after a short course of prednisolone by mouth, even when the dose was increased progressively to at least 60 (and in some cases to 1000) mg/day. Although nine were on regular treatment with a glucocorticoid aerosol, the mean $FEV_{1.0}$ for the whole group was no higher on the day of blood sampling than on earlier clinic visits.

Group 2: 15 asthmatic patients who were 'glucocorticoid sensitive', as shown by an increase in $FEV_{1.0}$ of more than 30% after a 7 day course of prednisolone in a dose of only 20 mg/day. Nine of these patients had recently started regular treatment with a glucocorticoid aerosol, and in all 15 the disease was well controlled at the time of blood sampling. This may explain why the mean $FEV_{1.0}$ was significantly higher at that time than at their initial clinic visit.

Group 3: 11 'new' asthmatic patients whose response to prednisolone, in terms of $FEV_{1.0}$, had not previously been assessed.

As observed in the original study [1], the glucocorticoid-resistant patients were on average older, had a longer history of asthma, and were more prone to nocturnal wheeze and 'morning dipping' than the sensitive patients. There was no difference between the two groups in respect of atopic status or smoking habits. The more frequent family history of asthma in the resistant patients noted in the original study [1] did not emerge in the smaller numbers included in the present series (Table 1).

The criteria for inclusion of patients in groups 1 and 2 eliminated all asthmatic patients whose $FEV_{1.0}$ response to glucocorticoid was in the 'grey area' between 15 and 30%, with the result that only patients who were unequivocally either 'glucocorticoid resistant' or 'glucocorticoid sensitive' were studied. Although this facilitated correlation of clinical and laboratory findings, it left unexplored the possibility that 'glucocorticoid resistance' and 'glucocorticoid sensitivity' might represent the ends of a continuous spectrum. It was hoped that the inclusion of group 3 would elucidate this point. The 11 sub-

TABLE 1. *Details of asthmatic patients and controls*

Values are means (± SD), or numbers of patients/total studied, unless otherwise indicated. *$P < 0.02$ (Mann–Whitney U-test).

	Group 1 (glucocorticoid-resistant asthmatic patients)	Group 2 (glucocorticoid-sensitive asthmatic patients)	Group 3 (new asthmatic patients)	Controls
Age (years)	55.7 (±17.0)	44.5 (±19.6)	58.2 (±12.9)	49.1 (±15.3)
Duration of asthma (years)	19.3 (±5.3)	11.5 (±5.3)	14.0 (±11.5)	–
Atopic	7/15	9/15	5/11	–
Nocturnal wheeze/morning dip	10/15	6/15	6/11	–
Family history of asthma	3/15	3/15	2/11	–
Smokers	3/15	3/15	2/11	–
Total dose of prednisolone in previous 12 months (mg)	205 (range 0–1200)	169 (range 0–700)	–	–
On regular treatment with steroid aerosol	9/15	9/15	–	–
$FEV_{1.0}$ (initial clinic attendance) (litres)	1.24 (±0.54)	1.59 (±0.71)	1.33 (±0.29)	–
$FEV_{1.0}$ (day of study) (litres)	1.21 (±0.58)*	2.48 (±0.79)*	1.33 (±0.29)	–
Plasma cortisol (day of study) (nmol/l)	506 (±140)	547 (±290)	535 (±150)	510 (±210)

jects in that group were asthmatic patients who had presented with moderately severe airflow obstruction for which a course of oral prednisolone was indicated. Their $FEV_{1.0}$ was measured before, and immediately after, a 7 day course of prednisolone (20 mg/day). Laboratory studies were undertaken in all 11 cases before treatment commenced and again in nine on its completion.

None of the asthmatic patients or the controls had coexisting malignant disease, was being treated with cytotoxic or immunosuppressive drugs, or was suffering from any form of intercurrent infection, including oropharyngeal candidiasis. About half of the patients in groups 1 and 2 had previously been treated with oral prednisolone but there was no significant difference between the groups in terms of total dosage. For at least 4 weeks before this study no subject had received glucocorticoid therapy other than beclomethasone dipropionate by inhalation. The dose did not exceed 400 μg daily, which is known not to suppress pituitary-adrenal function [3]. On the morning of blood sampling the mean plasma cortisol was normal in all groups (Table 1).

Analysis of blood samples

Peripheral venous blood (50 ml) was withdrawn at 09.00 hours from each subject into a sterile bottle containing 2 ml of 2% EDTA in phosphate-buffered sodium chloride solution (154 nmol/l). All the laboratory studies were carried out without knowledge of the clinical status of the subjects. Total leucocyte count, differential leucocyte cell count and plasma cortisol were determined on all samples.

MNC were obtained from the peripheral blood by centrifugation over Ficoll-Hypaque [4] and were then washed twice in Hank's balanced salt solution. Subpopulations of T-lymphocytes, monocytes and Ia-positive cells were quantified by means of monoclonal antibodies with the following specificities: OKT3 (peripheral blood T-cells), OKT4 (inducer T-cell subset); OKT8 (cytotoxic/suppressor T-cell subset), OKM1 (peripheral blood monocytes) and Ia231 (MNC bearing Ia antigens, including 90% of B-lymphocytes and monocytes, 20% of null cells, and activated T-lymphocytes). Antibodies in the OK series were obtained from Ortho Diagnostics Ltd.

Cells positive for OKT3, OKT4, OKT8, OKM1 or Ia231 were detected by indirect immunofluorescence using fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulin (Meloy Ltd) as the second antibody. The percentage of cells with membrane fluorescence was calculated from study of 200 cells. For each case a negative control was included: cells to which only second antibody had been added.

Colony formation from peripheral MNC

MNC from each subject were cultured by using previously described methods [5, 6]. For each culture, 2×10^5 cells were suspended in 40 μl of RPMI 1640 supplemented with 10% heterologous serum and 0.3% agar, and plated on to an underlayer of 0.2 ml of 0.5% agar containing 20 μg of phytohaemagglutinin (Difco) and 10% heterologous AB serum (the same source of serum was used throughout). Methylprednisolone sodium succinate (MP; Upjohn) was added to underlayers to give a final concentration of 10 nmol/l. Control cultures received no MP. All cultures were in 17 mm diameter wells in Multiwell dishes (Linbro) and were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air. After 5 days of incubation colonies (defined as aggregates of more than 40 cells) were counted by phase contrast microscopy at x 100 magnification in three central fields in each of three replicate wells. This relatively simple scoring method has been verified by detailed computer-assisted analysis of the size distribution of all cell clusters in the cultures. Detailed immunophenotyping of these colony cells showed them to consist mainly of OKT4 and OKT8 lymphocytes, but most colonies also included a central cell bearing monocyte markers (M. C. Poznansky, A. C. H. Gordon, I. W. B. Grant & A. H. Wyllie, unpublished work).

Results

Analysis of the peripheral blood leucocytes revealed that there were few differences between the known 'glucocorticoid-resistant' asthmatic patients (group 1) and the 'glucocorticoid-sensitive' asthmatic patients (group 2) or between the asthmatic patients and normal control subjects. As expected, the asthmatic patients had an eosinophilia not found in controls. The resistant and sensitive patients did not differ significantly from each other or from control subjects in terms of the proportion of T-cells, T-inducer cells, Ia-positive cells or cells reacting with the OKM1 monocyte monoclonal antibody. There was a slightly higher proportion of T-suppressor/cytotoxic cells in the blood of sensitive asthmatic patients as compared with controls. The mean proportion of T-suppressor/cytotoxic cells in resistant asthmatic patients was less than in sensitive asthmatic patients, but this difference failed to attain significance at the $P < 0.05$ level (Table 2).

TABLE 2. *Immunophenotype of peripheral blood mononuclear cells of asthmatic patients and controls*

Mean results ± SD are shown. *$P < 0.01$ (t-test).

Phenotype	Mean percentage of mononuclear cells		
	Group 1 (resistant asthmatic patients)	Group 2 (sensitive asthmatic patients)	Controls
OKT3	45.1 (±10.9)	46.2 (±7.0)	47.2 (±11.6)
OKT4	27.2 (±7.1)	26.0 (±7.9)	30.9 (±9.1)
OKT8	17.8 (±5.5)	21.6 (±6.3)*	16.3 (±4.7)*
Ia231	13.8 (±4.9)	13.5 (±6.2)	12.9 (±6.9)
OKM1	29.0 (±7.4)	26.7 (±10.0)	29.3 (±9.4)

TABLE 3. *Effect of glucocorticoid on colony formation by peripheral blood mononuclear cells from asthmatic patients and controls*

The reduction in colony number with methylprednisolone is significant for controls and sensitive asthmatic patients ($P < 0.0001$) but not for resistant asthmatic patients. Mean results ± SD are shown.

Culture conditions	Colony number		
	Group 1 (resistant asthmatic patients)	Group 2 (sensitive asthmatic patients)	Controls
No glucocorticoid	35.3 (±9.5)	38.3 (±6.6)	37.7 (±5.2)
Methylprednisolone	31.8 (±12.8)	13.8 (±7.3)	14.7 (±8.8)

In the absence of MP, MNC from both controls and asthmatic patients in groups 1 and 2 generated similar numbers of colonies in soft agar after 3–5 days incubation, representing a plating efficiency of approximately 0.05% (Table 3). Incubation during growth with MP at 10 nmol/l significantly inhibited colony numbers developing from the MNC of control subjects and sensitive asthmatic patients. In contrast this concentration of MP had little effect on colony development from the MNC of resistant asthmatic patients (Table 3).

To minimize purely technical variations in the plating efficiency and in order to permit comparison between patients, the number of colonies developing from each patient's MNC in the presence of MP at 10 nmol/l was expressed as a percentage of the number developing in its absence (Fig. 1). It was clear that the responses of individual patients, expressed in this way, fell with very few exceptions into two categories. Colony growth from MNC of glucocorticoid-sensitive patients was strongly inhibited by MP *in vitro*, whereas growth from MNC of resistant patients was affected little by MP. In all but one of the control subjects colony growth was inhibited by MP to the same extent as that from glucocorticoid-sensitive asthmatic patients.

The clinical sensitivity of the 11 asthmatic patients in group 3 to glucocorticoid was unknown at the outset of the study. In these patients, however, the response to glucocorticoid, as determined by colony inhibition *in vitro*, was found to correlate closely with the extent to which $FEV_{1.0}$ increased during subsequent treatment with oral prednisolone (Fig. 2). This is shown in Fig. 2, where percentage changes in colony number, induced *in vitro* by MP, are plotted against the percentage change in $FEV_{1.0}$ resulting from a 7 day course of oral prednisolone. An identical relationship was evident when the responses *in vitro* were plotted against the absolute change in $FEV_{1.0}$ (data not shown).

The percentage change in colony number, induced by MP *in vitro*, appeared to be characteristic for each subject. Thus, on re-testing six subjects between 3 weeks and 4 months later, the divergence from the initial value was in every case less than 20% (Fig. 3*a*). In particular, colony

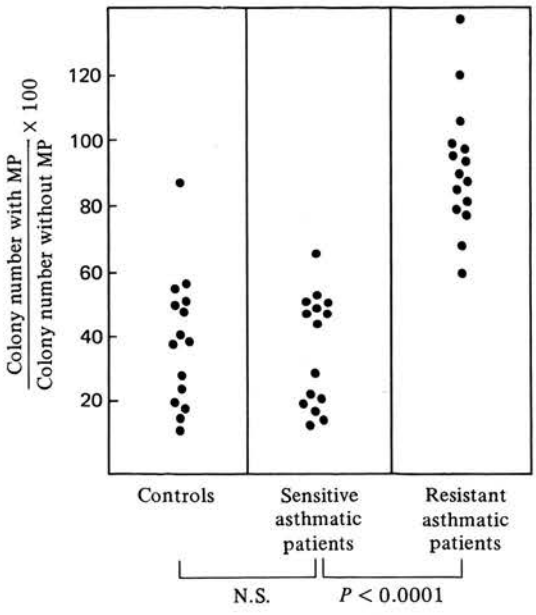

FIG. 1. Effect of methylprednisolone (MP) on colony formation from mononuclear cells of resistant and sensitive asthmatic patients (groups 1 and 2) and of controls. The number of colonies developing in the presence of methylprednisolone at 10 nmol/l is expressed as a percentage of the number which developed in the absence of methylprednisolone, from cells of the same subject. Each point derives from a different subject. N.S., Not significant.

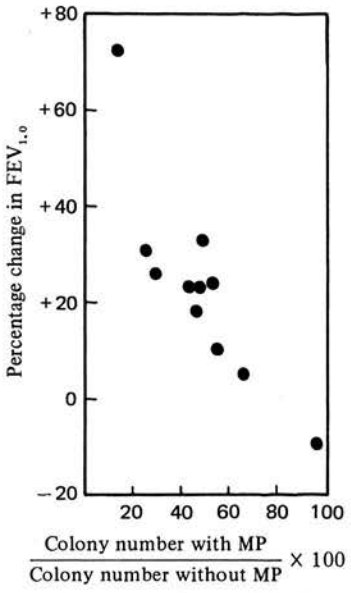

FIG. 2. Correlation between inhibition of colony growth by methylprednisolone (MP) *in vitro* and relief of bronchospasm by a course of prednisolone *in vivo*, in the 11 unselected patients in group 3. The correlation is significant ($P < 0.005$).

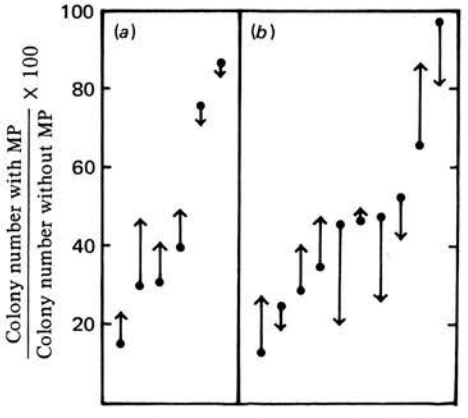

FIG. 3. Repeated estimations of inhibition of colony growth by methylprednisolone (MP) *in vitro*. In (*a*) the initial values (●) and values obtained on re-testing under identical conditions (point of arrow) are shown for six subjects (two of them glucocorticoid resistant). In (*b*) initial values (●) and values obtained immediately after a 7 day course of oral prednisolone (point of arrow) are shown for ten subjects, nine of which came from group 3 (new asthmatic patients).

numbers from glucocorticoid-sensitive patients and control subjects were strongly inhibited by MP on both occasions (i.e. to less than 60% of the untreated value). In glucocorticoid-resistant patients colony numbers remained in excess of 60% of the untreated value.

Perhaps surprisingly, glucocorticoid medication did not appear to affect the extent to which colony growth was inhibited by MP *in vitro*. Thus in the ten subjects tested before and immediately after the 7 day course of oral prednisolone, colony growth remained strongly inhibited in eight, and little affected in two (Fig. 3*b*).

Discussion

Previous studies of the characteristics of overtly glucocorticoid-resistant asthmatic patients have revealed few differences from the majority of patients with chronic asthma, who respond well to glucocorticoids [1, 2]. In this paper we show that MNC from such glucocorticoid-resistant patients were significantly more resistant to the effects of MP *in vitro* than the MNC from clinically sensitive asthmatic patients or normal controls. The criterion adopted was the ability of phytohaemagglutinin-stimulated MNC to grow as colonies in soft agar. This phenomenon is known

to depend upon interactions of monocytes and lymphocytes [6, 7], but as the proportions of monocytes and T-cells in the peripheral blood of the two groups of patients were similar, the observed difference in glucocorticoid responsiveness *in vitro* cannot be attributed merely to differences in the relative numbers of cell types under study.

MNC from individual patients with clearcut glucocorticoid-resistant or -sensitive asthma (i.e. groups 1 and 2) responded to MP *in vitro* in a consistent manner on repeated testing. The response bore no relationship to the endogenous plasma cortisol at the time of blood sampling (see Table 1). Further, the response *in vitro* did not change after a 7 day course of systemic glucocorticoid therapy. Hence it is probable that the MNC of each individual have a characteristic response to glucocorticoid *in vitro*, uninfluenced by endogenous cortisol or by the administration of exogenous glucocorticoid.

The close correlation between the response to glucocorticoid *in vitro* and *in vivo* suggests that the cell types involved in generating colonies *in vitro* (i.e. monocytes and T-lymphocytes) may be similar to those contributing to the pathogenesis of asthma in these patients. A substantial body of evidence implicates cells of the monocyte lineage in the pathogenesis of asthma [8–10]. There are several ways in which improvement in the clinical condition of asthmatic patients treated with glucocorticoids may depend upon the effects of these drugs on such cells. Of particular relevance to the interpretation of the present results is the well-documented inhibition of production of interleukin 1 by low concentrations of glucocorticoids [11–13]. Interleukin 1 is a monocyte product which mediates mitogen-induced T-cell proliferation *in vitro* [14], and is known to support T-cell colony formation in soft agar [7]. It is also a pyrogen and promotes the synthesis of inflammatory mediators from arachidonic acid [15, 16]. Other effects of glucocorticoids that may be implicated in their therapeutic action in asthma are modulation of complement receptor expression by monocytes [2] and induction in leucocytes of the phospholipase A_2 inhibitor lipomodulin [17], which also suppresses reactions mediated by immunoglobulin E [18]. It thus may be postulated that cells of monocyte lineage within the lungs of glucocorticoid-resistant asthmatic patients might continue to secrete inflammatory mediators, support lymphocyte proliferation and exist in a hyperreactive state with enhanced complement receptor expression, despite the presence of inhibitory concentrations of glucocorticoids.

We embarked on this study on the premise that glucocorticoid resistance in chronic asthma was an all or none phenomenon, which is what prompted us to restrict our investigations *in vitro* initially to patients whose asthma was readily recognizable on clinical criteria as sensitive or resistant. It would seem, however, from the observations on previously untreated, and thus unselected, asthmatic patients, that sensitivity and resistance may form a continuous spectrum. At one end would be patients whose asthma is overtly resistant to systemic glucocorticoids, even in high dosage. It is important to recognize these patients since they may otherwise be needlessly exposed to hazardous side effects. This study indicates one way in which such patients may be identified.

Although the cellular basis of glucocorticoid resistance remains obscure, our findings suggest that a defect in MNC, and perhaps monocytes in particular, may be responsible. We are currently defining the nature of this cellular defect more precisely. We are also exploring the potential of simpler tests *in vitro* to identify resistant patients before they are treated with glucocorticoids either for asthma or for other diseases normally responsive to these therapeutic agents.

Acknowledgments

A grant from Upjohn Ltd is gratefully acknowledged. The monoclonal antibody Ia231 was donated by Dr K. Guy, M.R.C. Clinical and Population Cytogenetics Unit, Edinburgh.

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